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STUDIES ON INDIAN SIMULIIDÆ

Part VIII.

FAR EASTERN ASSOCIATION OF TROPICAL MEDICINE 9TH CONGRESS

THE meeting of the above Association which was announced in the April 1933 issue of this *Journal* to be held at Nanking, China, from the 2nd to the 8th October, 1933, has been postponed by the Chinese Government. A further announcement as to where and when the 9th Congress will meet will be made in this *Journal* in due course. The notice in the April number may therefore be regarded as cancelled.

Editor

S. aureum), however, the hind basitarsus though not enlarged is not parallel-sided. The adults of both those species are characterized by having a coarse golden pubescence on the abdomen and their hypopygia show a great deal of resemblance with each other.

Simulium (Eusimulium) aureohirtum (1911)

S. aureohirtum was described by Brunetti (1911) from two males and two females collected from 'Umling, Assam (III-07)', and several females (including type female) from 'Kanara, Bombay (VIII-07)', all of which, according to him, are in the Museum at Pusa. In the collection of simuliids now in the Pusa Museum there are 14 specimens (6 females and 8 males) marked 'Umling, Assam, III-07, Patel', and 4 females and 2 males marked 'Kanara, Bombay, P. G. P. VIII-07' (types and some of the paratypes of *aureohirtum* being in the British Museum, London). Of the former 14 specimens, 7 males and 4 females belong to *aureohirtum*.

Brunetti, 1 male to *metatarsale* Brunetti, 1 female to *pattoni* Senior White and 1 female to *christophersi* Puri while all the specimens from Kanara, Bombay, belong to *S. pattoni* S W After examining the types and paratypes on which Brunetti had based his species Edwards (1928) has already pointed out that the males belong to one species and the females to two others, and that the name *aureohirtum* Brunetti must be retained for the male

Brunetti's description of the male as well as of the female is so vague that he himself was not able to identify his species properly The male specimen, selected by him as the neotype of *S. indicum* Becher, belongs to his species *aureohirtum* and among the specimens identified by him in 1917 for the Pusa Museum are two males and two females from Simla, all marked by him as *aureohirtum*, but an examination of these shows that they all belong to *S. aureum* Linneus which species is markedly distinct from *aureohirtum* Under the circumstances detailed descriptions of the male as well as of the female appear to be very necessary

FEMALE

Head dark slate, with short pale golden pubescence Frons moderately broad, somewhat narrowed in the region of the antennæ, its greatest width at the top a little less than one-third the width of the head The colour of the antennæ is variable that of the two basal segments varies from yellow to reddish orange and brown, while that of the rest from yellowish orange to reddish and dark brown The colour of the two basal segments is usually lighter than that of the rest of the antenna, and that of the third the darkest In majority of specimens the colour of the antennæ is reddish orange Palpi brownish black

Thorax —Mesonotum dull, greyish black, covered densely with a short, pale golden pubescence Some specimens show a pair of very narrow, sub-median greyish longitudinal lines Scutellum somewhat brownish black, with pale golden pubescence and having a row of long, pale golden, marginal hairs Pleuræ dark grey, membranous area as well as the mesosternum bare

Abdomen dull black dorsally, covered with a pale golden pubescence, basal scale pale yellowish brown, fringe of hairs golden Venter somewhat yellowish black *Terminalia* (Plate I, fig 1) Short and slender macrosetæ are evenly distributed on the posterior two-thirds of the ventral surface of segment 7, sternite of segment 8 is broad, somewhat thinly chitimized, with broadly rounded lateral ends Except on its anterior half and a narrow portion in the middle line, the sternite is covered with short slender macrosetæ The anterior gonopophyses are thinly chitimized and appear as broad projection of the posterior border of sternite 8, without any line of demarcation separating them from the sternite itself Their interno-lateral border is somewhat thickened, with the interno-posterior end rounded and produced backwards The paraprocts and cerci are thinly chitimized and of moderate size

Legs —Fore coxæ pale yellow, posterior ones nearly black, all trochanters pale yellow, the femora also pale yellow with their distal one-fifth or so black, the black pigment on the fore femora not as well marked as that on the posterior legs, that on the hind leg being the most conspicuous Fore tibiæ pale yellow with the distal one-fourth black and a faint sub-basal blackish yellow ring, middle and hind tibiæ pale yellow with the distal one-third black and fairly broad yellowish black

sub-basal band, that on the latter much better marked than that on the former. These black bands vary in their density but are invariably present and are characteristic of this species. Fore tarsi black, not flattened but cylindrical, the first segment over seven times as long as its greatest width. Segments 1 and 3 with a pair of long black hairs sub-apically on their posterior border. Base of the first tarsal segment of the middle leg, basal three-fourths of the first and the basal portion of the second tarsal segment of the hind leg pale yellow, the rest of tarsi black. Pedisulcus and calcipala well marked. All claws with a large basal tooth (Plate I fig. 2). All legs with fine pale golden pubescence, more conspicuous on the yellow than on the black portions.

Wings—Normal, hyaline, radius hairy throughout. Radial sector simple. Wing length about 2.7 mm.

Halteres yellowish white.

Buccal cavity without any cluster of nodules or teeth on its ventral or dorsal surface. The posterior border of the ventral surface regular and more or less straight.

The arms of the furca curved downwards, their ends fixed to a triangular chitinization, not as well developed in species in which segment 8 bears a large tergite. *Spermatheca* single, oval in form and uniformly dark brown. It differs from those of most of the other species examined so far in not having a rounded colourless area in the region of the commencement of the duct, on the other hand a short basal portion of the duct is formed of the thickened dark brown chitin which appears to line the wall of the spermatheca (Plate I, fig. 3).

MALE

Head black, with a fringe of short black hairs. Face pale grey with sparse golden hairs. Antennae varying in colour from yellow to reddish orange and dark brown. The colour of the third basal segment is darker than that of the rest of the antenna and is usually dark brown. Palpi brownish black.

Thorax—Mesonotum when viewed from in front appears dull greyish black, with a broad velvet black median longitudinal stripe, about one-fourth the width of the mesonotum, gradually widening out posteriorly and having a very narrow pale grey lateral border*. When viewed from behind the whole of mesonotum appears velvet black with a pair of narrow, greyish, sub-median longitudinal stripes. It is covered uniformly and fairly densely with a short golden pubescence, somewhat copper coloured on the median velvet black area, and pale golden to silvery near the anterior border on either side. In certain lights the lateral as well as the posterior borders of the mesonotum appear slate grey. Scutellum varies from brownish black to black, is covered with a short golden pubescence and has a fringe of long golden hairs. Pleurae slate grey, membranous area as well as the mesosternum bare.

Abdomen velvet black, with a coarse golden pubescence, long hairs on the basal scale black basally, their distal two-thirds golden. A pair of silvery spots present on segment 2 dorsally but the spots usually present on segments 5-7 in

* In some specimens there is a faint grey, very narrow median stripe as well

the males are absent in this species, a slight greyiness being observable laterally along the posterior border of segment 7 only. *Genital armature* (Plate I, fig 4) is markedly different from those belonging to the sub-genus *Simulium*. The coxites are comparatively large, longer than their greatest width near the base, bearing a number of macrosetae, three or four of which are much longer than the others. The styles are very short, only about half the length of the coxites and are somewhat rectangular in shape (Plate I, fig 5), their width a little shorter than their length. Their distal end is somewhat concave and each style bears a short sub-terminal spine on its dorsal surface near its outer edge. The inter-coxal piece (Plate I, figs 4 and 6) is broad, plate-like, somewhat folded in the middle, with a median keel-like process projecting downwards from its ventral surface. This process is densely covered all over with long conspicuous setae. The pair of apodemes at the anterior ends of the inter-coxal piece are short, slender and curved. The median chitinous plate lying above and a little behind the inter-coxal piece is long and narrow with a round posterior end. The lateral chitinizations of the mesosome, just behind the coxites, are comparatively narrow and appear like a long spine on either side. Instead of the cluster of spines found on each side of the genital opening in the sub-genus *Simulium* there is only a single large spine on each side. The region behind the genital opening is covered with irregular rows of fine setae arising in groups. The cerci are in the form of a pair of thinly chitinized flattened processes directed inwards and a little backwards (Plate I, fig 4c) and the paraprocts are inconspicuous protuberances at the bases of the cerci.

Legs —Coxae, trochanters and femora as in female. All tibiae yellow basally, gradually becoming black on the distal half and with a well-marked, broad, sub-basal black ring (Plate I, fig 7). Fore tarsi black cylindrical, first segment over seven times its greatest width. Basal half of the first and the base of the second tarsal segments of the hind leg blackish yellow, the rest of middle and hind tarsi black. The hind basitarsus (Plate I, fig 7) not enlarged, practically parallel-sided. It is about three-fourths as long as the hind tibia (0.78 of the length of the latter) and proportionately as broad, its greatest width being 0.75 of that of the latter and only 0.23 of its own length. The yellow portions of the legs bear golden pubescence.

Wings as in female

PUPA

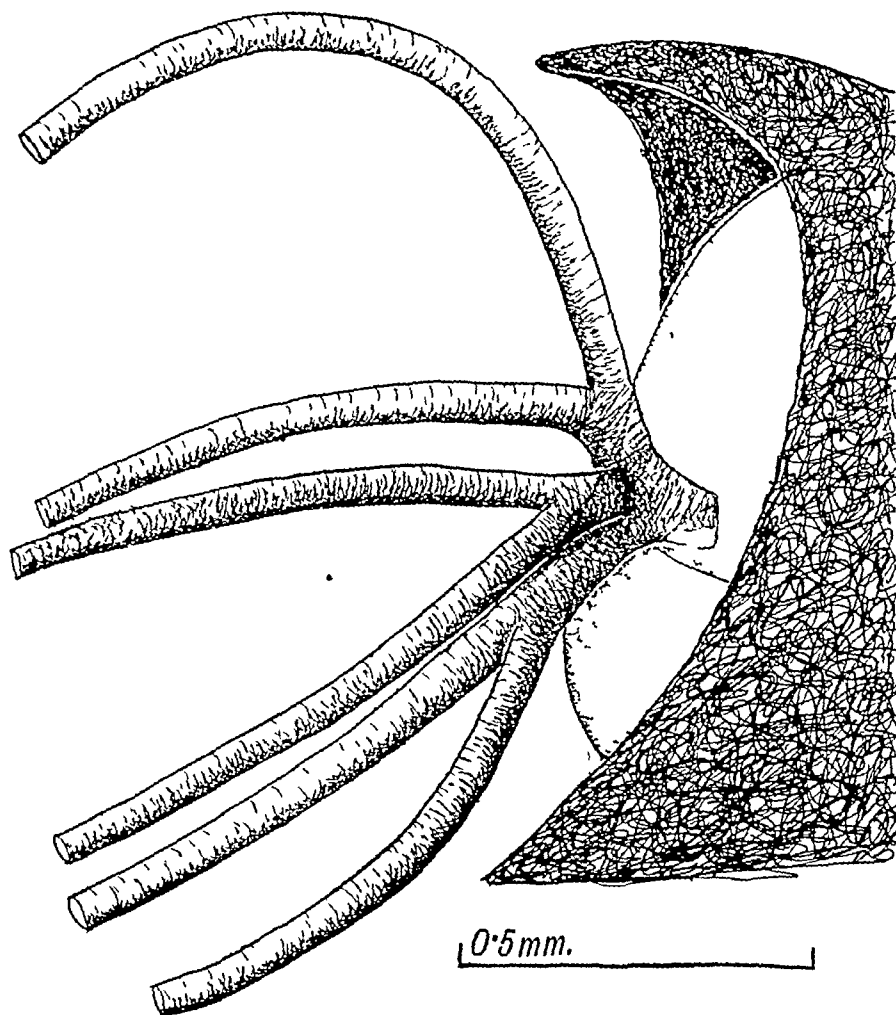
Size about 2.7 mm × 1.0 mm

The integument of the head and thorax is light brown, sparsely covered with moderate-sized disc-like tubercles. The head bears the usual three pairs, while the thorax has five pairs of trichomes dorsally. These trichomes are short and simple. The cuticular hooks on the dorsal as well as the ventral surface of the abdomen are as in *himalayense*, with one or more strongly chitinized sensory hairs on the ventral surface of segment 4. Dorsally on segment 8 there is an irregular row, broken in the middle line, of backwardly directed cuticular spines along the anterior border, a few* spines present on segment 7 and some very minute ones on

* The number of these spines is very variable in this species

segment 9 also. The pair of sub-terminal spines on segment 9 are well developed.

Respiratory filaments (Text-figure) are a little longer than the pupa, 6 in number, arranged in three stalked pairs—a ventral, an outer and a dorsal pair.



TEXT FIGURE

I M PURI del

Simulium aureohirtum BRUNETTI

Pupal respiratory filaments of left side *in situ*. Anterior end of pupa and cocoon drawn to show mode of origin of filaments.

The stalks are comparatively stout and are widely divergent, as are also the filaments. The surface of the filaments is covered with minute tubercles, somewhat

larger ones placed on the ridges forming a reticular pattern while smaller ones sparsely covering the interspaces, as in *milgmicum*

Cocoon is light yellowish brown to dirty brown in colour, of the ordinary wall-pocket shape, fairly closely woven, without any windows or interspaces in the mesh. Its anterior border is not thickened and bears a short median rather broad anterior process at its dorsal end. It practically covers the pupa, its length along its base being about 3.7 mm. and dorsally up to the end of the median process about 3 mm. The width of the cocoon often varies from about 1.4 mm. to 2.4 mm.

LARVA (FULL-GROWN)

Average length about 5.6 mm., colour varying from dirty cream to pale brownish yellow without any definite markings.

Head golden brown, usually with four dark fairly well-defined spots on the dorsal surface, two elongated, median and one on each side near the posterior border of the frons-clypeus. The posterior and larger of the two median elongated spots is usually the darkest of all. The poorly chitinized area on the ventral surface of the head-capsule not very wide, extending anteriorly to even less than half the length of the head. *Antennæ* long and slender, 1-segmented, 1st segment about 6 times as long as broad, 1st three segments are about equal in length, and 4th is minute. *Cephalic fan* filaments number about 40. On the *mandibles* (Plate I, fig. 8) the cluster of bristles behind the terminal teeth are arranged as in *aureum**. The number of teeth in the sub-terminal row on the ventral surface appear to be variable but usually there are 6 teeth gradually decreasing in size. The pair of tooth-like processes on the inner border are about equal in size and shaped as in the figure. *Sub-mentum* (Plate I, fig. 9) has 9 teeth in the terminal row and 5 on each side. The median tooth and the one at each end of the front row are comparatively much larger than the others and the 3rd tooth from each end is the smallest. There are 5 long hairs on each side on the ventral surface of the sub-mentum.

Each of the three anal gills have three to five branches near the base, decreasing in size distally. *Posterior sucker* has about 60 rows, with 10-13 hooks in each. Ventral papillæ moderately long and conical.

DISTRIBUTION

This species was originally described from two males and two females from Umling, Assam (11-07), and in the Pusa Museum collection now there are 8 males and 3 females from Umling, Assam (15-11-07, Patel's), a number of females from Igatpuri, Nasik and Sonagad Forest (all in Bombay Presidency), 11-11. I have bred out over 800 specimens (males and females) most of them from isolated pupæ collected from the following places —

The Punjab — Large numbers from Shamri Nala (below Bishop Cotton School), Simla, 21-vii-26, a small stream crossing Kasauli-Subathu Road, viii-26, R Ghagai near Pinjaur, Patiala State (6-iv-30), on grass in the River Leh at and near Rawalpindi, 9/15-xi-30, a few in the stream at Wah (near Hasanabdal), Punjab, 11-xi-30.

* See C Text figure 17 (Puri, 1925)

The Madras Presidency —Stream near Dhobighat, Coimbatore, 9-1-28, large numbers on submerged grass in a stream near Dhobighat, Ootacamund (10-1-28), small stream below waterworks, Vizagapatam (9-11-28), a single specimen from a stream near Tanmalai Railway Station, Tinnevelly District, 21-1-31

The Bombay Presidency —Small streams near Manmad, Nasik, Igatpuri, and Kasara 10/11-11-30, a few specimens from near Poona, 23-11-30, a small stream below Dhobighat, Mahabaleshwar, 23-11-30, stream below the Reservoir at Lonavla, 25/26-11-30, small streams near Savantvadi (Savantvadi State), Sirsi and Gersopa 29-11-30/3-1-31

Cooing —Large numbers from small seepage streams in Mercara (8-1-31)

Assam —Large numbers on grass in a small stream joining the large one near Race Course, Shillong, 14/15-1-32 large numbers in a small stream (seepage) near Margherita, and in the River Lamdang between Margherita and Ledo, 22-1-32

Practically in all the streams in which this species was found breeding, the water was not very clean, and the grass blades, as also the larvæ and pupæ of this species, were covered with a very fine muddy deposit. The water was usually contaminated with soap washings and flowing close to human habitation.

As pointed out already by Edwards (1928), *aureohirtum* Brunetti closely resembles *S. diversipes* Edwards (1928a), described from Rodriguez. According to him, *aureohirtum* differs from the latter mainly in broader male claspers (styles) and also in the more golden and almost unicolorous mesonotal pubescence in both sexes.

Dr Edwards has very kindly sent me a male and a female of *diversipes*, collected from Natal. After a detailed comparison of these two specimens with those of *aureohirtum* I am unable to find any difference in the coloration of the mesonotal pubescence in the two species. The male of *diversipes* (from Natal) differs from that of *aureohirtum* only in having a comparatively longer style which is also comparatively narrow at its base, gradually widening out distally. The females differ only in that the spermatheca has no dark brown neck in *diversipes*, as is invariably present in the other species.

***Simulium (Eusimulium) aureum* FRIES**

Simulium aureum Fries is common in the whole of the palearctic region extending down to the Mediterranean sub-region, and from India it has so far been collected only from Simla and the surrounding hills in the western Himalayas. Edwards has already given brief descriptions of the adults* as well as of the larvæ and pupæ (1921) of this species from England and Petersen (1924) has described them briefly from Sweden, but neither of these authors have dealt with the terminalia of the female. Moreover, as in North-West India this species occurs together with a number of other closely allied ones, with which it is apt to get confused, I have given below a detailed description of the adults (of *S. aureum*) incorporating that given by Edwards.

* Under *S. augustipes*

FEMALE

Head blackish grey, with golden pubescence (which is present on the frons as well as the face) *Antennæ* black with the two basal segments varying from orange to reddish *Palpi* black

Thorax —Mesonotum dull, blackish grey, densely covered with a bright somewhat pale golden pubescence *Scutellum* brownish black, covered with a golden pubescence and has a fringe of long golden hairs *Pleuræ* bare In some specimens mesosternum with a few golden hairs near its lateral ends

Abdomen brownish black, densely covered with pubescence similar to that present on the thorax *Terminalia* closely resemble those of *aureohirtum*, differing from them only in certain details The short slender macrosetæ on the ventral surface of segment 7 are comparatively closely placed in the middle third and often have a few somewhat longer setæ arising among them The anterior gonopophyses extend comparatively much more backwards and the median gap between the two is in some specimens much smaller than that found in *aureohirtum*

Legs —All femora and tibiæ pale yellow with the distal one-fifth or so black, the anterior surface of the fore tibiæ with a somewhat silvery sheen owing to a silvery pubescence *Tarsi* black except the basal three-fourths of the first and the base of the second tarsal segment of the hind leg *Fore tarsi* not flattened, hind basitarsus not enlarged, parallel-sided All claws with a large blunt basal tooth All yellow parts of legs with golden pubescence

Wings normal, radius hairy throughout, wing length is about 3.1 mm *Halteres* pale lemon yellow

Arms of the furca fairly stout, the angle between them not sharply defined

Spermatheca as in *aureohirtum* but the neck comparatively shorter

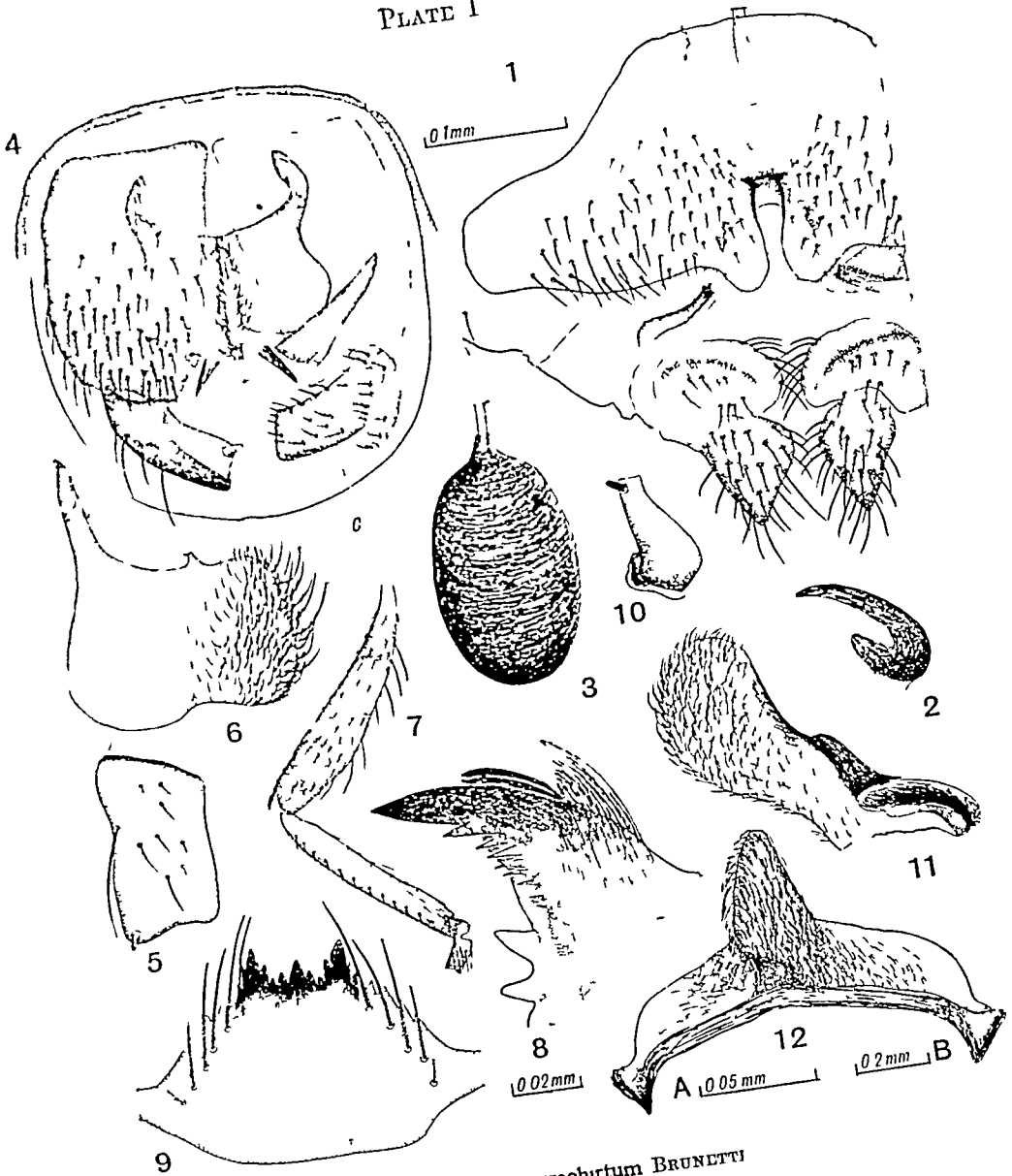
MALE

Head —Face with slender golden hairs, antennæ black

Thorax —Mesonotum velvet black, more or less densely covered with golden pubescence, somewhat dark brown in the middle line near the anterior border *Scutellum*, pleuræ and mesosternum as in female

Abdomen velvet black, with coarse golden pubescence dorsally Long hairs on basal scale golden *Genital armature* (Plate I, figs 10–12) resembles to some extent that of *aureohirtum* The coxites are rather large, broadest about their middle, gradually narrowing down distally The styles are only about half as long as the coxites, twisted upwards a little above their base, distal end with a somewhat hammer-shaped tip and a single spicule The inter-coxal piece has a narrow, somewhat thinly chitinized base with the pair of apodemes comparatively long and stout and a median keel-like process projecting downwards and forwards from its ventral surface As in *aureohirtum* this process is densely covered all over with long conspicuous setæ and in most of the specimens its distal end is somewhat swollen The lateral chitinizations of the mesosome and the spines on each side of the genital opening, are as in *aureohirtum* Paraprocts inconspicuous, cerci large, directed inwards, somewhat conical distally The imbricate rows of fine setæ in the region behind the genital opening are comparatively more conspicuous

PLATE I



I M PURI del

Simulium aureohirtum BRUNETTI

- Fig 1 Ventral view of part of terminalia of female (Scale as in Fig 8)
 " 2 A claw from hind leg of female (Scale as in Fig 8)
 " 3 Spermatheca of female (Scale as in Fig 8)
 " 4 Ventral view of genital armature of male Left coxite drawn in outline only Left style not shown (Scale as in Fig 1)
 " 5 Ventral view of right style of male (Scale as in Fig 1)
 " 6 Lateral view of inter coxal piece of male (Scale A)
 " 7 Tibre, basitarsus and second tarsal segment of left hind leg of male (Scale B)
 " 8 Ventral view of the distal end of left mandible of male (Scale as in Fig 1)
 " 9 Dorsal view of sub mentum of larva (Scale as in Fig 1)

Simulium aureum FRIES

- Fig 10 Ventral view of style of left side of male (Scale as in Fig 1)
 " 11 Lateral view of the inter coxal piece of male (Scale as in Fig 1)
 " 12 Antero ventral view of the inter coxal piece of male (Scale as in Fig 1)

Legs brownish black except the basal half of middle and hind tibiae which are distinctly yellow. Fore tibiae often appear silvery on their anterior surface owing to pale pubescence on them. Tarsi blackish. Hind basitarsus moderately enlarged. Its greatest width about its middle is a little less than one-fourth its own length and about 0.73 of the greatest width of the hind tibiae, its length being 0.7 of that of the latter. Golden hairs on the yellow portions of the legs, the rest covered with a fine black pubescence with numerous long black hairs scattered all over.

Wings as in female.

For detailed descriptions of pupæ and larvæ see Puri (1925), pp. 354-56.

Material collected — This species has so far been collected from around Simla only. There are 2 males and 3 females in Pusa Museum marked 'Simla, x-11' and I have bred out a few specimens from isolated pupæ collected from streams crossing Simla-Kalka Road above Kandaghat, 27-viii-29, and the stream north of Chhota Simla, September 1930.

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STUDIES ON INDIAN SIMULIIDÆ

Part IX

S EQUINUM VAR *MEDITERRANEUM* PURI AND *S PARÆQUINUM* SP N

BY

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THE two species described in this paper are characterized by having the basal section of the radius hairy, front tarsi not flattened, tibiæ without any silvery dusting, tip of abdomen of female dull and claws of female simple, without any basal tooth. According to the classification proposed by Enderlein (1930) these species belong to the genus *Wilhelmia*, sub-family *Nevermanniinae* differentiated from *Simulinae* on the cylindrical (not flattened) front tarsi. Owing to the presence of the simple claws in the female, Edwards (1923), following Roubaud's classification, places *S equinum* in the sub-genus *Simulium* separating it from *Simulium*, S Str, into a separate group *Wilhelmia* (Enderlein), because of the presence of cylindrical fore tarsi in the adult and peculiar respiratory organs in the pupa. Later, however, Edwards (1931) has again sunk *Wilhelmia* under *Simulium* (S Str) saying that the amount of widening of the fore tarsi was very variable in the different species and it would often be difficult to say whether the flattening was present or not.

A detailed study of the adults of *equinum* and *paræquinum* has shown that the only characters in which they indicate any resemblance to the sub-genus *Simulium*, so far as the Indian species are concerned, are the presence of simple claws in the female and in having the vein R₂₊₃ completely bare above. While, on the other hand, the presence of well-defined cerci in the male, the general structure of the male and female hypopygia and the absence of any teeth or nodules at the posterior end of the buccal cavity, the posterior border of which is complete, isolate them completely from the sub-genus *Simulium*. All these characters indicate their

close relationship to the sub-genus *Eusimulium*. The claws of the female are very distinctive and unlike any other in either of the sub-genera. Moreover, the epipharynx in the female and the structure of the median plate-like chitinization of the mesosome, lying dorsal and close to the inter-coxal piece in the male, are markedly different from those of any other species belonging to either of the two sub-genera and these three characters coupled with the peculiar respiratory organs of the pupa make it advisable to put these species in a separate sub-genus, *Wilhelmia* (Enderlein), as originally proposed by Edwards.

The respiratory organs of *Simulium nodosum* (Puri) are no doubt of a somewhat similar nature as those in these two species but the flattened fore tarsi, the presence of a strong buccopharyngeal armature, claws though simple of moderate size only, separate it from the sub-genus *Wilhelmia*.

***Simulium* (*Wilhelmia*) *equinum* VAR *mediterraneum* PURI**

Edwards (1921) first pointed out that the adults of *S. equinum* from the Mediterranean region differed from the type form, common in the British Isles and North Europe, in the paler colour of the thoracic pubescence in the female and later (Edwards, 1923) drew attention to the fact that their pupæ too differed from those of the type form. The Mediterranean form was subsequently described by me (1925) as a distinct variety—*mediterraneum*, from species collected from Redcyef, Tunis and El Outay, Algeria giving a detailed description of the pupa. A comparison of the specimens of *S. equinum* occurring in North-West India with those of the type form collected in England has shown that the former undoubtedly belong to the variety *mediterraneum*, differing from the latter mainly in the pupal stage.

Edwards (1915) has given brief descriptions of the adults of the type form from the British Isles but unfortunately he has not described the female hypopygium and that of the male too has not been dealt with in detail. As another species very closely allied to *S. equinum* occurs in North-West India, differing from the latter mainly in the structure of the genital armature in the male and to some extent in the ornamentation of the thorax in the male as well as in the female, it seems advisable to give a detailed description of the adults of the variety *mediterraneum*.

FEMALE

Head dull grey, sparsely covered with somewhat silvery pubescence. Antennæ varying in colour from yellowish or reddish brown to nearly black, with the two basal segments yellowish to reddish brown. Palpi black.

Thorax—Mesonotum dull pale grey, with three narrow, rather ill-defined, blackish longitudinal lines, the sub-median pair usually not reaching the anterior border, somewhat narrowed and approximated to each other anteriorly, gradually broadening out posteriorly and converging a little in the prescutellar region where all the three lines meet. The whole of the mesonotum sparsely covered with a somewhat slender pale pubescence which is more silvery than golden. Scutellum is dark grey, densely covered with a coarse very pale golden pubescence and a large number of long pale golden hairs forming a fringe. Pleuræ dark grey, with the

membranous area between the prothoracic stigma and the wing-base covered with slender pale golden hairs

Abdomen nearly black, densely covered with a coarse pubescence of the same colour as on the thorax, long hairs on the black basal scale pale golden. *Terminalia* (Plate II fig 1) Short slender macrosetæ scattered over the ventral surface of segment 7, lying somewhat closely on the middle fourth. Sternite of segment 8 fairly broad shaped as in figure, somewhat depressed in the middle, slender macrosetæ scattered on most of its surface, except on the depressed area. *Anterior gonopophyses* thinly chitinized, somewhat triangular in shape, with the posterior end long, drawn out and curled upwards (Plate II, fig 2). Paraprocts, fairly strongly chitinized, rather large with few macrosetæ, cerci comparatively small

Legs —All coxæ and trochanters black, femora and tibiæ vary in colour from yellowish black to black, their distal end always black and the base of the tibiæ always pale yellow. All tarsi black except the basal two-thirds of the first and the base of the second tarsal segments of the hind leg which are pale yellow. Front tarsi cylindrical, first segment more than 7 times as long as its greatest width, segments 1 and 3 with a pair of long black hairs sub-apically on their posterior border. Pedisulcus well marked, calcupala of moderate size. All claws simple (Plate II, fig 3), much larger as compared to the size of the insect. All legs with sparse pale golden pubescence not as well marked on the tarsi as on the rest of the leg

Wings normal, hyaline, radius hairy throughout. Radial sector simple, concave vein, bearing setæ only on its under-surface and none above. Wing length varies from 2.0 mm to 2.9 mm. In specimens collected during the summer (June) from South Waziristan (N-W F Province) the average length is 2.1 mm, while in those collected during November from around Rawalpindi (Punjab) it is 2.8 mm

Halteres pale lemon yellow

Buccal cavity without nodules or teeth on its ventral or dorsal surface. Its posterior border smooth and more or less straight. Epipharynx (Plate II, fig 4) with a long chitination broadening out posteriorly on its ventral surface, and bearing a number of fine chitinous spicules in a patch near its posterior end. Arms of the furca rather slender, their posterior end fixed on each side to a large triangular chitination. Spermatheca single, globular, dark brown, the area round the origin of the duct, colourless

MALE

Head —Face dark grey with creamy silvery pubescence. Antennæ black, with the two basal segments yellowish black

Thorax —Mesonotum more or less velvet black, its anterior one-third, except for a moderately narrow middle portion, appearing dull pale grey when viewed from in front. Some specimens show three narrow, ill-defined, pale grey longitudinal lines on the anterior two-thirds of the mesonotum. The whole of the mesonotum covered with pale golden pubescence, denser and paler on the anterior greyish portion and in front of the scutellum. Scutellum dull black, covered with coarse pale

golden pubescence and having a fringe of long similar coloured hairs. Membranous area of the pleuræ with slender hairs as in the female.

Abdomen velvet black, sparsely covered with fine pale golden hairs and with clusters of longer ones laterally on segments 3-5. The usual silvery spots present on segments 2 and 5-7 but they are somewhat greyish. *Genital armature* (Plate II, fig 5) Coxites very large gradually narrowing distally. Styles arising sub-terminally, small, narrow, somewhat curved, directed upwards and forwards when at rest. Each bears a single, broad papilla-like spicule at its narrow distal end (Plate II, fig 6). The inter-coxal piece is of the shape of a wide V, the limbs rather broad and ending in fairly stout apodemes, the tip of the angle slightly bent downwards and a little forwards and bearing on it a number of conspicuous slender setæ, a few present also on its ventral surface. The median plate lying above and ending close to the inter-coxal piece is fairly strongly chitinized, rather short and moderately broad, its posterior end modified into a pair of thickened knobs bearing strong teeth (Plate II, fig 7). The lateral chitinizations of the mesosome narrow, ribbon-like. A cluster of spines present on each side in the region of the genital opening, but all the spines, unlike those found in the sub-genus *Simulium*, arise from a single (rounded) plate-like structure. The area between the genital opening and the posterior end of the median plate is studded with fairly strong short chitinous spicules. Paraprocts inconspicuous, cerci well marked, somewhat narrowed distally.

Legs black, knees narrowly yellowish, basal two-thirds to three-fourths of first and the base of the second tarsal segment of the hind leg pale yellow (the rest of tarsi black). Front tarsi cylindrical. Hind basitarsus not enlarged, parallel-sided, narrow. It is about three-fourths as long as the hind tibia and about 7 times its own width which is nearly half that of its tibia. All the legs with a coarse pale golden pubescence.

Wings as in female.

PUPA

The pupa of this variety has already been described in detail. The trichomes are short and simple, disc-like tubercles are very minute and few in number. Rows of cuticular spine on the dorsal surface of abdomen are absent. The pair of sub-terminal spines very small, usually absent. Pairs of hook-like spines on ventral surface of abdomen very strong on segments 6 and 7 but less so on segment 5, segment 4 only occasionally bears a small more or less strongly chitinized sensory hair.

The main difference between this variety and the type form lies in the structure of the respiratory organs which are in the form of thin walled tubes. From its origin the organ extends upwards and downwards as a broad tube lying entirely in contact with the pupa on the one side and with the thickened rim of the cocoon on the other. Unlike the condition found in the type form the dorsal and the ventral ends of this basal section narrow down gradually and are continued along the ends of the basal section of the opposite side thus forming a complete circle on the dorso-anterior surface of the pupa. From the middle of this basal section are given off 6 long, somewhat slender minor branches, three from the internal and three from the dorso-external surface. They arise more or less close together, the lowermost and

also the longest of the outer three often arising a little apart from the others, all spreading out distally. The bases of these minor branches show a somewhat ringed appearance.

Cocoon resembles that of the type form somewhat boot-shaped, 3.7 mm × 1.6 mm in size.

LARVA

The larva of the variety resembles that of the type species and has already been described in detail*. In the specimens collected from India the usual dark spots on the head are present but often not sharply defined.

DISTRIBUTION

I have bred out a large number (over 100) of specimens, both males and females, from isolated pupæ collected from the following places —

A large number of larvæ and pupæ on submerged grass blades in a large irrigation channel on the road between Sarvakai and Jandola, South Waziristan, N.-W. F. Province (30-v-1928), on grass in Ghagar stream, near Pinjaur, Patiala State (6-iv-30), large numbers on grass blades in River Leh, a stream near Khanna and on stones in River Sohan, all near Rawalpindi (Punjab) (10/15-xi-30).

A few specimens were bred out by Colonel Sir Rickard Christophers, C.I.E., O.B.E., F.R.S., I.M.S., from pupæ collected from a stream near Prang, Kashmere (September 1930).

Simulium (Wilhelmia) paræquinum SP. N.

This species closely resembles *S. equinum* var. *mediterraneum* differing from the latter only in certain details which alone have been given below, (the main difference in the two lies in the structure of the male genital armature).

FEMALE

The frons is dull grey with a narrow median ill-defined dark line dividing it into two. The thoracic pubescence is comparatively sparse and the blackish lines much better developed than in *S. equinum*. The sub-median lines reach the anterior border, each ending anteriorly in a broad dark spot. The hypopygium closely resembles that in the other species except that the sternite of segment 8 is comparatively poorly chitinized and its outline is not as well defined as in the latter.

MALE

The mesonotum is dull black and usually does not show any greyness in the antero-lateral regions. The pubescence is golden instead of pale golden or nearly silvery as found in var. *mediterraneum*.

* See pp 356-57, and Text figure 18 (Puri, 1925a)

Genital armature shows certain amount of resemblance to that described for *S. sergenti* Edwards. The coxites (Plate II, fig 8) are comparatively large somewhat rounded distally. The styles are placed much further from the tip of the coxites and are narrow, curved upwards, their distal end somewhat truncate (Plate II, fig 9), the papilla-like spicule is much smaller and is sub-terminal in origin. The inter-coxal piece (Plate II, fig 10) is comparatively stout, somewhat U-shaped, with the bottom much broader than the arms, its ventral surface bearing a large number of conspicuous setæ in the posterior region. The median plate (Plate II, figs 10 and 11) has a peculiar form, lies above the inter-coxal piece and is moderately broad. Posteriorly it is somewhat notched bearing a large number of teeth on its two rounded ends. From the middle of the posterior border of the median plate arises a strongly chitinized deeply grooved projection, directed upwards and a little backwards, the like of which I have not seen in any other species so far. The spines forming a cluster on each side of the genital opening arise from an elongated plate (Plate II, fig 12), one spine near the anterior end being comparatively very long.

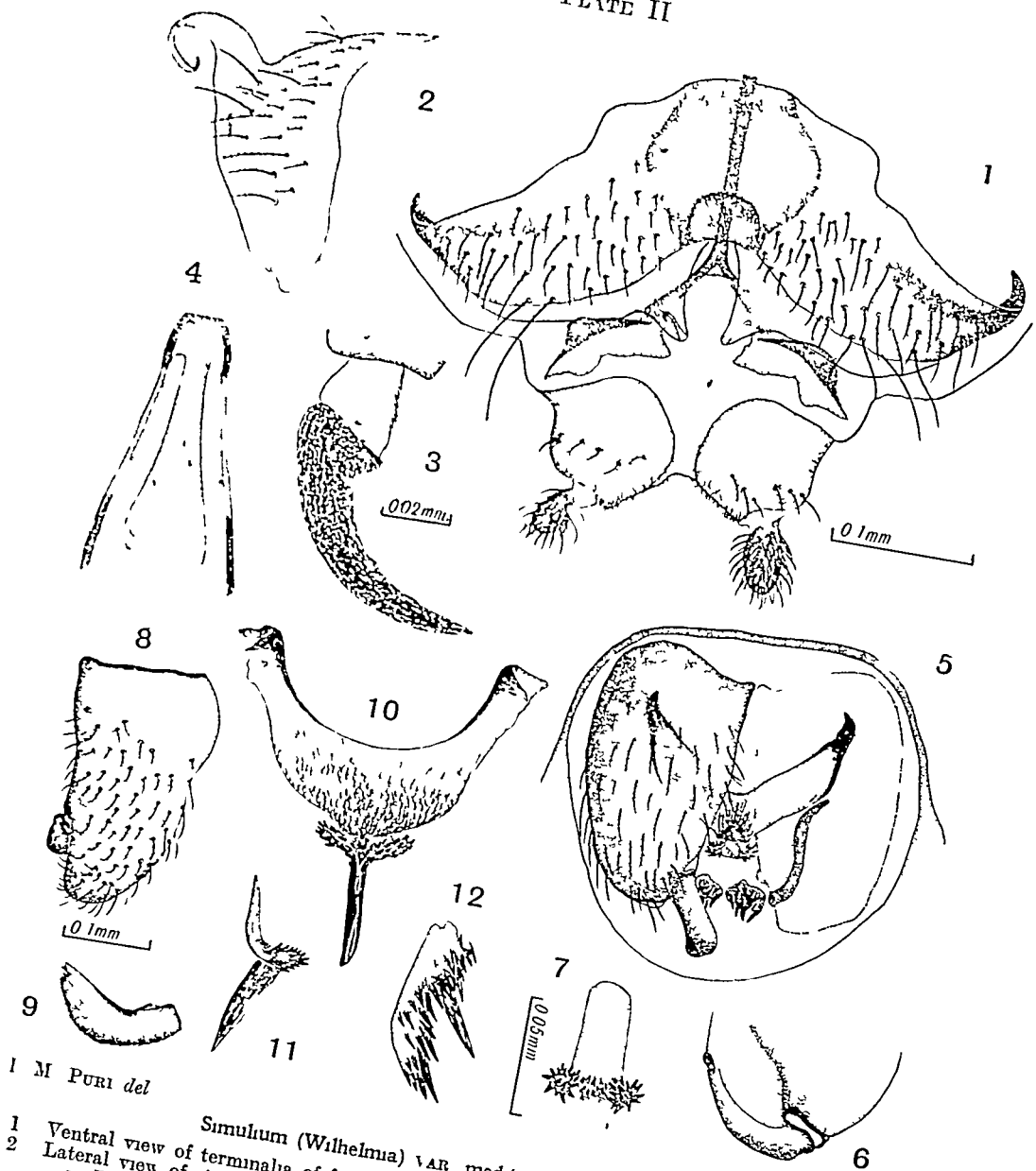
Neither the larvæ nor the pupa appear to have any noticeable distinction from those of *S. equinum* var. *mediterraneum*.

Described from a large number of males and females (over 200) all in good condition and bred out of isolated pupæ collected from a large stream at Wah near Hasanabdal (District Campbellpore), Punjab (11-xi-30).

Types and paratypes in my own collection.

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Simulium (Wilhelmia) var. mediterraneum PURI

- Fig 1 Ventral view of terminalia of female
 " 2 Lateral view of terminalia of female
 " 3 A claw from hind leg of female (Scale as in Fig 1)
 " 4 Hypopharynx of female (Scale as in Fig 1)
 " 5 Ventral view of genital armature. Left coxite drawn in outline only. Cerci and left style not shown of male (Scale as in Fig 1)
 " 6 Dorsal view of distal end of right coxite showing the style of male (Scale as in Fig 1)
 " 7 Median plate like chitination of the mesosome of male (Scale as in Fig 1)

Simulium (Wilhelmia) paræquium sp. n.

- Fig 8 Ventral view of left coxite of paratype male
 " 9 Lateral view of right style of paratype male (Scale as in Fig 1)
 " 10 Ventral view of inter coxal piece, also showing the median chitination of mesosome of paratype male (Scale as in Fig 1)
 " 11 Lateral view of median chitination of mesosome of paratype male (Scale as in Fig 1)
 " 12 Cluster of spines on the left of the genital opening, showing the plate from which they arise of paratype male (Scale as in Fig 1)

A PROTEOLYTIC ENZYME IN CUCUMBER (*CUCUMIS SATIVUS*)

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CUCUMBER is one of the commonest of fruits and is said to have been cultivated in India for about three thousand years. It is extensively used for edible purposes and religious ceremonies and certain parts of this plant have been largely used in medicine. The leaves, boiled and mixed with powdered cumin seeds, are administered in throat affections. The seeds, powdered and mixed with sugar, are believed to have powerful diuretic properties and are sold in the bazaars of Upper India under the name of ' tukhmi-khiyara ' . In febrile conditions the soles of feet are rubbed with cucumber. In sunstroke pieces of cucumber are put in the bed with the idea that inhalation of the emanations from the fruit will cool down the heat of the body. According to Atkinson the juice is said ' to banish wood lice and fish-insects by strewing freshly cut thin slices in their haunts ' .

We took up the investigation of this fruit on account of the popular belief in northern India that eating of cucumber is associated with attacks of cholera. Many of the severe and fatal type of cases are said to have occurred in those who have eaten cucumber previously. In other parts of the country this fruit is said to help digestion if taken after meals. These facts led us to investigate the possibility of cucumber containing certain enzymes which might be responsible for these effects. The pioneer work of Vines has shown that erepsins are widely distributed in the plant kingdom. Out of a variety of materials—fruits, leaves, stems, bulbs, tubers, etc—studied by Vines, only a few failed to show ereptic activity.

The proteolytic enzyme of *Cucumis melo* was studied by Hou and Chen (1927). Ambros and Harteneck (1929) mention the presence of a protease in *Cucumis sativus* and classified the plant proteases obtained from various sources into distinct types.

PREPARATION OF THE EREPTIC SOLUTION FROM CUCUMBER

The fruits used were those grown in August and September in India and, unless otherwise mentioned, fresh mature fruits were used for these experiments. The skin was first removed and the fruit was cut in thin slices, thoroughly macerated in a porcelain mortar and the resulting pulp was squeezed through linen. The juice thus obtained was then filtered through cotton-wool.

The juice of the mature fruit was acid in reaction. 10 c.c. of the juice requiring 1 c.c. to 1.2 c.c. of N/10 NaOH to neutralize the acidity, with phenolphthalein as an indicator.

Attempts to concentrate and purify the enzyme by adsorption with kaolin and alumina and also precipitation with acetone proved ineffective. We had therefore to perform our experiments with the expressed juice. The juice obtained from different fruits varied somewhat as regards their proteolytic action, so proper controls were run with each.

DIGESTION OF WITTE'S PEPTONE

A 5 per cent solution of Witte's peptone in 1 per cent saline was used as the substrate, 10 c.c. of the juice was mixed with 10 c.c. of the peptone solution and the volume made up to 21 c.c. with the addition of distilled water. Digestion was carried on at 39°C–40°C for 21 hours. Toluene was used as a preservative in this and other subsequent experiments. The extent of digestion was measured by the Van Slyke's amino-nitrogen estimation method and also by the depth of colour as obtained by the bromine-water test for tryptophane.

TABLE I

Mark on the tubes	Experiments	Temperature and duration of incubation	Mg. of NH_2 -nitrogen per c.c. of the mixture	Tryptophane test
1	10 c.c. peptone solution + 10 c.c. juice + 1 c.c. H_2O	21 hours at 39°C–40°C	1.04	Strong
2	Do + 10 c.c. boiled juice + 1 c.c. H_2O	Do	0.78	Negative

It will appear from Table I that the juice of *Cucumis sativus* contains a fairly strong protease which breaks down Witte's peptone into tryptophane and other amino-acids.

CUCUMBER PROTEASE IS OF EREPTIC NATURE

Inability to digest fibrin and the power to split off tryptophane from Witte's peptone was considered by Vines as a criterion for judging the ereptic properties of a particular material. Accordingly, fibrin washed free from blood with normal saline and also Merck's fibrin from blood stained with Congo Red were allowed

to digest with cucumber juice, acid or alkali being added as indicated in Table II Digestions were carried on at 39°C–40°C for six days

TABLE II

Mark on the tubes	Experiments	Temperature and duration of incubation	Tryptophane test
1	A small flocl of fibrin + 5 cc juice	Six days at 39°C–40°C	Negative
2	Do + 5 cc boiled juice	Do	Do
3	Do + 5 cc juice + 0.5 cc N/10 HCl	Do	Do
4	Do + do + 0.5 cc N/10 NaOH	Do	Do
5	Congo Red fibrin + 5 cc juice	Do	Do

No digestion of the fibrin was observed in any of the tubes This shows that the protease in the cucumber juice is ereptic in nature

OPTIMUM REACTION OF THE MEDIUM

The following experiments were done to determine under what conditions of acidity or alkalinity the enzyme has the optimum activity —

TABLE III

Mark on the tubes	Experiments	Temperature and duration of incubation	Initial pH of the mixture	Mg of NH ₂ —Nitrogen per cc of the mixture	Tryptophane test
1	10 cc peptone solution + 10 cc juice + 1 cc H ₂ O	Done immediately after mixing	6.8	0.74	Negative
2	Do + do + do	21 hours at 39°C–40°C	6.8	1.01	Strong
3	Do + do + 1 cc N/10 HCl	Do	5.8	1.13	Very strong
4	Do + do + 1 cc N/1 HCl	Do	3.0	0.95	Faint but distinct
5	Do + do + 1 cc N/5 citric acid	Do	5.6	1.11	Very strong
6	Do + do + 1 cc N/10 NaOH	Do	7.4	0.97	Almost the same as No 4
7	Do + do + 1 cc N/10 Na ₂ CO ₃	Do	7.2	0.95	Do
8	Do + do + 1 cc HCN (B.P. dil 2 per cent solution)	Do	5.4	1.12	Very strong

It will be seen from Table III that cucumber erepsin acts best in a slightly acid medium. The optimum activity appears to be between pH 5.4 to pH 6.2. A fairly strong acid reaction on the other hand inhibits its activity considerably. A faintly alkaline reaction has the same effect as adding excess of acid.

OPTIMUM TEMPERATURE

Cucumber erepsin has been found to manifest its maximum activity at 39°C–40°C. Temperatures higher or lower than this have the effect of retarding the activity.

DIGESTION OF CASEIN

Plant erepsins are not usually considered as capable of hydrolysing casein like animal erepsins. But it will be apparent from the following experiments that cucumber erepsin like 'cabbage erepsin' (Blood, 1910-1911) does hydrolyse casein —

The casein solution was prepared by rubbing up in a mortar 4 g of casein with 30 c.c. of N/10 NaOH, diluting this to 120 c.c. with distilled water and finally filtering through cotton-wool.

TABLE IV

Mark on the tubes	Experiments	Temperature and duration of incubation	Mg. of NH_3 —Nitrogen per c.c. of the mixture	Tryptophane test
1	10 c.c. casein solution + 10 c.c. juice + 1 c.c. H_2O	Done immediately after mixing	0.35	Negative
2	Do + do + 1 c.c. H_2O	Five days at 39°C–40°C	0.64	Strong
3	Do + do + 1 c.c. N/10 HCl	Do	0.78	Stronger than No. 2
4	Do + do + 1 c.c. 2 per cent HCN (B.P. dil.)	Do	0.79	Almost the same as No. 3

LIQUEFACTION OF GELATINE

Cucumber erepsin has the property of liquefying gelatine. A 5 per cent melted gelatine containing 0.1 per cent thymol was used as the substrate. To 2 c.c. of the gelatine solution amounts of cucumber juice as indicated in Table V were added, the volume made up to 4 c.c. in each case with distilled water and incubated at 39°C–40°C.

TABLE V

Mark on the tubes	cc gelatine solution taken	cc cucumber juice added	cc H ₂ O added
1	20	03	17
2	20	05	15
3	20	10	10
4	20	20	
5	20		2

After incubation at 39°C–40°C for 21 hours, only No 4 remained liquid on cooling but after 72 hours' incubation all the tubes excepting No 1 and No 5 failed to solidify on cooling

RENNETIC ACTIVITY

The question whether rennetic activity is due to the existence of a distinct enzyme or is merely the first stage of the manifestation of proteolytic action is not still quite settled, but it appears from the following (Table VI) experiments that the cucumber juice possesses the power of clotting milk as well

Unboiled milk freed from fat with chloroform and kept in the cold storage for about a week was used. The milk was amphoteric in nature when the experiments were started. The cucumber juice was carefully neutralized before it was mixed with the milk

TABLE VI

Mark on the tubes	Experiments	Temperature of incubation
1	2 cc milk + 03 cc juice + 17 cc H ₂ O	39°C–40°C
2	Do + 05 cc juice + 15 cc H ₂ O	Do
3	Do + 07 cc juice + 13 cc H ₂ O	Do
4	Do + 10 cc juice + 10 cc H ₂ O	Do
5	Do + 20 cc juice	Do

There was no sign of clotting in any of the tubes within six hours. The tubes were therefore left in the incubator overnight. After incubation for 22 hours Nos 5, 4 and 3 were found to have clotted almost completely. After 48 hours there was clotting in No 2 also. The tubes were left in the incubator with the

addition of fresh toluene from time to time but no tryptophane could be detected even in No 5 after a week's digestion

ACTIVATION WITH HCN

The activity of the cucumber ereptase is much enhanced by the addition of HCN but it is doubtful whether this activation is specific for hydrocyanic acid or it is merely due to the acid reaction. On referring to Table III it will be seen that 1 c c of N/10 HCl or 1 c c of N/5 citric acid activated it to almost the same extent. These experiments were repeated several times but in no case could we find any specific activation with HCN both qualitatively and quantitatively.

THE MESOCARP AND THE ENDOCARP

In order to see whether the ereptic enzyme is uniformly distributed or differs in activity in the different parts of the fruit, the juice was expressed separately from the mesocarp as well as from the endocarp of the same fruit and the relative activity of these was tested on Witte's peptone solution, casein solution, etc.

The results of the digestion of peptone solution only are given in Table VII

TABLE VII

Mark on the tubes	Experiments	Temperature and period of incubation	Mg. of NH_2 -Nitrogen per c c of the mixture	Tryptophane test
A	10 c c peptone solution + 10 c c juice + 1 c c N/10 HCl	Done immediately	0.85	Negative
A ₁	Do	21 hours at 39°C - 40°C	1.07	Strong
B	10 c c peptone solution + 10 c c juice + 1 c c N/10 HCl	Done immediately	0.87	Negative
B ₁	Do	21 hours at 39°C - 40°C	0.91	Faint

A = juice expressed from the endocarp B = juice from the mesocarp

The results indicate that the ereptic activity of the endocarp is decidedly greater than that of the mesocarp and this difference in activity is noticeable also as regards digestion of casein solution, rennetic activity and the liquefaction of gelatine.

MATURE AND IMMATURE FRUITS

In the course of these experiments immature fruits were sometimes used and as these yielded a lower amino-acid value on digestion with peptone and gave a fainter

tryptophane test than was usual with mature fruits, the effect of the degree of maturity on the ereptic activity was studied. It was found that the activity of erepsin in mature cucumber is greater than either of that in immature or ripe fruits as judged both from Van Slyke figures and intensity of the tryptophane coloration.

The fruits in which the seeds have hardened but the skin has not assumed that rusty brown colour and cracked appearance are regarded as mature.

Erepsins are stated to have their optimum activity in a slightly alkaline reaction (pH about 7.8). Cucumber erepsin behaves differently in this respect in requiring a slightly acid reaction for its maximum activity. During an epidemic of cholera people are afraid of taking cucumber as they believe that its use will make them susceptible to the disease, and in a number of severe cases of cholera a definite history of the patient having ingested cucumber previous to the attack was obtained. We do not of course attribute the attack of cholera to the use of cucumber alone but there seems to be some relationship between the two. In the light of the existence of a fairly strong ereptic enzyme in cucumber it may be suggested as an explanation that this enzyme probably reinforces the proteolytic activity of cholera vibrio breaking down the proteoses and peptones formed from the intestinal epithelium with the production of increased amounts of toxins. Cucumber is also known to help digestion if taken after meals. Since this enzyme can act in an acid medium it can carry the digestion of the proteins to the amino-acid stage even in the stomach which peptic digestion alone cannot accomplish.

SUMMARY AND CONCLUSIONS

1. A fairly strong ereptic enzyme is present in cucumber which hydrolyses Witte's peptone and casein with the formation of tryptophane. It has also the property of liquefying gelatine and clotting milk but it has no action on fibrin.

2. This enzyme acts best in a slightly acid solution (pH 5.4 to 6.2) but a fairly strong acid reaction or a slightly alkaline reaction retards its activity considerably.

3. The endocarp has a higher concentration of the enzyme than the mesocarp.

4. The enzymatic activity is stronger in the mature fruits in which the seeds have hardened than in the immature or ripe ones.

5. Hydrocyanic acid activates this enzyme considerably but this action does not appear to be specific as in the case of papain or bromelain, as it is activated to almost the same extent by dilute HCl or citric acid.

6. An explanation of why ingestion of cucumber prior to an attack of cholera increases its virulence and why it helps digestion if taken after meals has been suggested.

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STUDIES ON THE PHYSICAL PROPERTIES OF DIFFERENT BLOOD SERA

Part V

BUFFER ACTION

BY

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NAPIER (1924) has shown that the buffer action of the blood from kala-azar patients is lower than that of the blood from normal persons. The method employed in the determination of pH values is a combination and modification of the colorimetric methods suggested by Dale and Evans (1920) and Evers (1921). An appreciable portion of the acid added was probably adsorbed by the red blood corpuscles (Christophers 1929) and this part of the acid had therefore obviously no share in bringing about the change of pH of the plasma. Moreover, it is unlikely that normal and pathological red blood corpuscles would adsorb hydrochloric acid to the same extent. In view of these considerations it would be of interest to study in a comparative way the buffer action of normal and kala-azar sera. Incidentally the buffer action of syphilitic and leprotic sera has also been determined.

EXPERIMENTAL

The pH value of the serum has been determined by the hydroquinhydrone electrode as developed by Bulmann and Lund (1921). These authors recommend 0.1 g of quinhydrone and 1 g of hydroquinone (quinol) for 15 c c of the fluid. The platinum wire of the electrode must be almost completely immersed in the precipitate, the reaction in such an electrode consists in the transformation of stable quinhydrone into stable quinol.

The e m f of the chain

Pt/quinol, quinhydrone, solution A/KCl/solution A/H₂/Pt, according to Bulmann and Lund is equal to 0.6179 volts at 18°C. The e m f of the same determined by us at the room temperature (26°C to 27°C) is 0.6174 volts. The pH value of the unknown solution can thus be determined from the formula

$$\text{pH} = \frac{E + 0.3734}{0.059}$$

where E is the e m f of the chain

Hg/Hg₂Cl₂/Sat. KCl/Sat. KCl/soln., quinhydrone quinol/Pt, and T is the absolute temperature, and taking 0.244 volts at the same temperature to be the e m f of the chain,

Hg/Hg₂Cl₂/Sat. KCl/Sat. KCl/HCl (H ion conc = 1 N) H₂/Pt when readings were taken at other temperatures, corresponding changes in the values dependent on T were done

One and a half to three c c of physiological salt solution was poured into a small electrode vessel and a mixture of dry quinhydrone and quinol in quantities more or less such as has been mentioned above was added, the contents of the vessel were stirred and then 1.5 c c of serum was added, 1 c, diluted from 1:1 to 1:2 and then the contents of the vessel were stirred again, and the potential of this chain was read. The pH value calculated from this e m f has then been corrected by an amount ΔpH .

where $\Delta\text{pH} = 0.24 \times 2^{\text{pH}-7} - 0.21$, as suggested by Grossmann (1927). The results are given in Tables I to IV.

TABLE I
Normal persons

Pure serum	pH VALUE	
	0.6 c c serum + 0.4 c c 0.01 N HCl	0.4 c c serum + 0.6 c c 0.01 N HCl
7.26	6.95	6.75
7.28	6.98	6.79
7.35	7.05	6.80
7.38	7.07	6.86
7.32	6.99	6.79
7.37	6.99	6.76
7.39	6.97	6.86
7.34	6.95	6.78
7.33	6.98	6.84
7.27	6.94	6.79
Mean 7.329	6.98	6.814

TABLE II
Kala-azar

Pure serum	pH VALUE	
	0.6 c c serum + 0.4 c c 0.01 N HCl	0.4 c c serum + 0.6 c c 0.01 N HCl
7.43	6.89	6.65
7.34	6.96	6.66
7.37	6.90	6.65
7.37	6.98	6.70
7.45	7.02	6.57
7.31	6.90	6.63
7.20	6.89	6.68
7.20	6.83	6.65
7.37	6.90	6.67
7.34	6.88	6.63
Mean 7.34	6.91	6.64

TABLE III

Leprosy

Pure serum	pH VALUE	
	0.6 cc serum + 0.4 cc 0.01 N HCl	0.4 cc serum + 0.6 cc 0.01 N HCl
7.38	6.97	6.88
7.44	7.10	6.89
7.36	6.95	6.82
7.40	7.12	6.91
7.45	7.10	6.88
7.38	6.94	6.81
7.44	7.09	6.90
7.42	7.10	6.88
7.44	7.13	6.92
7.48	7.10	6.95
Mean 7.42	7.069	6.88

TABLE IV

Syphilis

Pure serum	pH VALUE	
	0.6 cc serum + 0.4 cc 0.01 N HCl	0.4 cc serum + 0.6 cc 0.01 N HCl
7.29	6.85	6.62
7.30	6.83	
7.33	6.79	6.62
7.28	6.94	6.70
7.21	6.83	6.58
7.32	7.00	6.72
7.35	6.94	6.71
7.40	6.99	6.78
7.45	7.01	6.85
7.38	6.95	6.81
Mean 7.331	6.88	6.71

DISCUSSION

From an analysis of these results and comparing them with those of Napier's, it is evident that a much greater quantity of hydrochloric acid (about 25 per cent *N/10* HCl) is required to produce an equal amount of change in the pH value of whole blood than that required for serum alone (about 6 per cent *N/10* HCl). It is therefore probable that an appreciable amount of the acid added was adsorbed by the red blood corpuscles and this amount had no share in bringing about the equilibrium between the hydrogen ion concentration of the whole blood and the dialysate.

Next comparing the changes produced by the same amount of hydrochloric acid with different sera we see that the buffer action of the pathological sera we have studied (kala-azar, syphilis, leprosy) is appreciably diminished and that the change is most marked in the case of kala-azar serum, a result in agreement with that of Napier's. From these data as well as those gathered on viscosity and surface tension (Chopra and Chaudhury, 1928, 1929*a*, 1929*b*) it would appear that pathological sera are generally characterized by higher viscosity, lower surface tension, and diminished buffer action.

A tentative suggestion for this diminution of buffer action in all pathological sera might be put forward in view of our results on the iso-electric points of at least one of the component proteins in the serum from the blood of kala-azar patients. Our observations on the gelation of kala-azar serum with formalin (Chopra and Chaudhury, *loc cit*) have shown that the iso-electric point of the gel-protein is at a pH of 7. Thus the serum with more of gel-protein will lose more of its power to neutralize acids and bases. This suggestion not only explains the loss of the power of kala-azar blood to neutralize acids and bases but also points strongly to the

correctness of our previous proposition that kala-azar serum contains a new protein—a gel-protein with iso-electric point at near about 7

SUMMARY AND CONCLUSIONS

It has been shown that the buffer action of different pathological sera is diminished and that the change in kala-azar is most marked

These results can be satisfactorily explained on the basis of the view that there is a gel-protein present in kala-azar serum whose iso-electric point is near about 7

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AN ANALYSIS OF SEVEN YEARS EPIDEMICS OF PLAGUE INVOLVING 2,520 INFECTED VILLAGES IN THE BELGAUM AND DHARWAR DISTRICTS, BOMBAY PRESIDENCY

BY

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- A Prediction of 'carrying over'
- B Probability of importation
- C Phases of the plague year
- D Differences in plague incidence in two tracts

SUMMARY AND CONCLUSIONS

APPENDICES

- A Form in which plague records were kept in the Bombay Presidency
- B Inquiries regarding the epidemics in certain villages with a view to determining whether they 'carried over'
- C Interpretation of records
- D Index to the numbered places of Chart 1

INTRODUCTION

THE Indian Plague Commission (1910) was the first authority to suggest that bubonic-plague epidemics are usually (though not always) lighted up from the smouldering embers of an infection the flames of which had not been quite quenched during the preceding epidemic season, in other words lighted up from a smouldering subterranean rat-enzootic* The Commission termed the phenomenon 'carrying-over'

As, however, it was observed that only a small proportion of villages infected in any year 'carried-over' to the next, Greenwood (1910, 1911) attempted to

* Recent work calls into question this hypothesis, but the attempted expression in this paper of the conditions under which 'carrying over' occurs is not prejudiced by any consideration of the actual mechanism that is at play

evaluate the factors concerned in the frequency with which they did so, and concluded that it was mainly an affair of large villages

Then the phenomenon was studied at Poona by Kunhardt and Chitre (1921) who, by investigating particularly the 'bridges', or places recording plague continuously throughout an 'off-season' (for definition of this term *see* p 36), concluded that it would be possible to predict with accuracy whether a place had any chance of carrying-over, and that chance was a function of its population and its date of 'primary infection'. As for the districts around Poona the chances were stated to be as given in Table I —

TABLE I

Kunhardt's formula for the prediction of carrying-over

There is some chance of carrying-over in a place —

with a population of over	and primarily infected by the beginning of
25,000	November
10,000	December
4,000	January
1,800	February
800	March
0	April

Now in this connection it is necessary to have a clear idea of the meaning of 'the primary infection'. It may be defined as the first human case dependent on a local rat-epizootic; an imported human case not caused by a local rat-epizootic would not be called a 'primary infection', it might be called a contingent infection. One must make the distinction as the two types exemplified have different epidemiological consequences. In the former case we presume a subterranean epizootic, in the latter this develops, if at all, much later, so that the consequential epidemic is also much later.

The hypothesis outlined above was subsequently applied by Kunhardt and Chitre to the practice of plague prevention, the operations being confined to the so-predicted carrying-over villages. It was believed that if plague could be eradicated in a matter of, say, 20 to 30 of such foci the resurgence of the great district epidemics of the Bombay Presidency would also be to a great extent, if not entirely, prevented. The great economy of such a plan over the usual practice of attempting to sterilize all places that had become infected during a year on the assumption that any might carry-over, can easily be realized.

The Indian Plague Commission, however, thought that carrying-over was responsible only for some plague-epidemics. Another way in which an epidemic might arise in a previously sterile area was shown to be from the importation, or

purely adventitious introduction, of infection, the Indian grain-trade being, with reason, chiefly blamed for this happening

Such doctrines were the principles of the scheme named the Belgaum and Dharwar Districts Plague Operations that were devised by the late Lieut-Colonel F G Hutchinson, C I E, I M S, as Public Health Commissioner with the Government of India, and Lieut-Colonel W O'S Murphy, I M S, Director of Public Health, the Government of Bombay, and placed in the writer's charge

The very essence of this scheme closely followed Kunhardt and Chitre which, as stated above, staked all on 'carrying-over' being *almost entirely* responsible for the recrudescence of the annual epidemics, though some provision was also made for the prevention of 'importation' into the larger grain centres. A great effort was to be made, after marking down by Kunhardt's formula the places likely to carry-over, to stamp out the disease in them during the off-season for if but one place eluded these efforts the plan would fail, such a single focus in the plague-season being able to do practically as much harm as a number in fact nothing would do but complete success as Dr Fabian Hirst (in litt) has remarked, 'it is obvious that if failure ensues in a single place it will cause the breakdown of the whole scheme'

It is not the purpose of the writer here to question the validity of the carrying-over theory, for the purpose of the following report he postulates its truth just as it must have been accepted by the authorities of the Governments concerned who drew up the scheme. The validity of Kunhardt's formula as such is similarly not called into question. It suffices that for the purposes of the operations the theory was thought to warrant the sanction of a practical trial, such as that here being discussed

However, when the operations came to be conducted it was found that *judging the epidemics by the same standards* (whether these were right or wrong) as they had been judged by Kunhardt in the Poona Districts, his formula was inadequate* for the purpose in view. For instance, the following records of plague-incidence (Table II) showed that certain places carried-over, although by the formula they would not have been predicted to do so —

TABLE II

Place infected	Taluka	Commencement of infection	Population	Sequel
Sampgaon	Sampgaon	January 1917	2,885	Carried over
Manoh	Khanapur	February 1917	910	Carried over
Asoge	Khanapur	February 1916	360	Carried over

* Vide the writer's Report on the Plague Operations in Belgaum and Dharwar Districts for 1920, Bombay Government Printing Press, Poona (1922) (Strickland, 1922), on places which had 'not been marked down as likely to carry over but which nevertheless developed reinfection due probably to carrying over' (page 11)

The realization occasioned by such cases was the mainspring of this 'analysis of seven years epidemics of plague in the Belgaum and Dharwar Districts' which it was hoped would reveal the exact circumstances under which carrying-over in these districts does take place, for without such knowledge of course we could not, as stated above, hope to succeed

Incidentally the inquiry led to conclusions cognate to the main thesis on matters which were of practical importance. The subjects dealt with will be discussed under the following headings —

- A Prediction of carrying-over
- B Probability of importation
- C The phases of the plague-year
- D Variations in plague-incidence in the two districts

Material for the analysis and its interpretation

The materials used for the analysis were taken from the records of plague-incidence entered in the office of the Director of Public Health, the Government of Bombay, from February 1913 to June 1920, and related to the infection of 2,520 villages with a population of less than 3,001

These records showed in the form such as is attached in Appendix A the two supposed factors of supreme importance, viz, the population and the month of first human plague-infection. The periods April to June, and July to the following March were respectively called the off-season, and the plague-season (for a discussion on these periods see Section C)

In the records, however, many errors gradually came to light. These were chiefly due to a very general tendency on the part of village-officers to hold back as long as possible any report of the onset of an epidemic, and it was obvious that a correct interpretation of what had happened in a place could not be arrived at from data vitiated in this way. Another constant difficulty inherent in the situation was that the populations given were those of the 1911 Census, and therefore in most cases different at the time of the epidemics that were (1917-1922) analysed.

It was only, however, when an *important* conclusion depended on the data that they were scrutinized and perhaps a village visited*, before accepting the records as good. For instance, the population of Bedag, Taluka Athni, was found to be 200 more than that shown in the register while Asoge, Taluka Khanapur (Chart 1, Block VII, No 194), was proved to have become primarily infected on 23rd March instead of in February, as in the records, both cases being 'critical' for the purpose of the analysis.

The interpretation of the records, even when correct, was not always easy, particularly as regards the date of onset of an epidemic, the date of an imported case might be confused with the date of the true commencement of the epidemic, however, if inquiry elicited the fact that several cases had occurred within the space of a few days or that the infection lay in houses and streets close together, it was usually possible to assign the commencement of the epidemic to a particular date

* See Appendix B for the results of visits to such villages

It must be reiterated that if the original case in a village was ascertained to have been imported the date of onset of the epidemic was held to be not that of the imported case, but that of the first case presumed to have been consequent on a rat-epizootic started independently of the imported human case for example, the case-record of this village —

TABLE III

Dec	Jan	Feb	Mar	Apr	May	June	July	Aug
2		4	3	1	3	11	53	37

The December infections were found to have been in women who came to the village to die from a neighbouring town then heavily plague-stricken, and although they may have brought the fleas which originated the rat-epizootic, or rats come independently, upon which the recorded epidemic from February to August was consequent, the onset of the primary infection was considered to have been in February and not in December

When one had obtained reliable data in a place regarding its population and date of primary infection, the next point to decide was whether, in the event of it showing infection during the next plague-year, it had 'carried-over' or had been reinfected. This, it must be pointed out, entailed a purely arbitrary judgment. One had to presume the *local* continuance or discontinuance of enzootic plague during the off-season with no other evidence than the incidence of the human cases, and what one could hear about them. There were no other criteria* available or possible to collect under the circumstances, and it could only be hoped that the results of the operations would justify the means.

Many of the considerations brought to bear on the interpretation of the records are given in Appendix C, in which some interesting cases of the apparent supervention of reimportation on a carried-over epidemic are discussed (*see* Appendix C, para 7)

ACKNOWLEDGMENTS

Before now proceeding to discuss the various sections of the analysis, the writer wishes to record his best thanks to the members of his staff during the operations, and particularly to Assistant-Surgeon S L Chinchankar, for his willing and valuable help in the somewhat tedious compilations involved. Grateful thanks are also due to Miss Clare Cameron of Queensland who, in the plotting of the charts, rendered great assistance.

A Prediction of carrying-over

In opening this section it must be strongly insisted that the operations necessitating this analysis were based on the postulate that carrying-over was the main

* There were no records of rat plague, and even if there had been, an apparent absence of it would not have proved it was absent

factor in the recrudescence of the district epidemics, and that if a single place carried-over the off-season during the operations, they could be said to have failed

The basic principles of the formula that carrying-over in an infected place was a function of its population and date of primary infection (*see* p 30) were accepted but as there had been the indication that Kunhardt's formula did not hold for these districts the point to be determined was under what circumstances of its population and date of first infection had a place *any* chance of carrying-over, and it must here be well remarked that the main object of the analysis was to find when a place had *any* chance of carrying-over. As a place once it had carried-over was of great danger, this was very important. If a place had *any* chance at all of carrying-over, it did not matter very much what were the odds

Method—For the analysis of the problem—when had an infected place *any* chance of carrying-over—the graphic method was employed as recommended by Kunhardt and Chitre (*loc cit*)

It involved the charting (*vide* Chart 1) of every plague-stricken village according to its size and date of first infection, using a dot to represent it, and numbering the dot if the village became reinfected

The records of each place so represented were then scrutinized, in order to decide by means of the usual criteria (*see* Appendices B and C) whether it had carried-over, and if there was any doubt its number on the chart was but half-ringed, whereas if it seemed certainly to have carried-over its number was completely ringed

When the 2,520 infected villages had been thus represented, a curve was drawn to demarcate those that had carried-over from those that had not done so

Then, as the position of the curve was the critical object of the analysis the records of the places represented *near the line* were re-examined, particularly because (1) it may have been decided that villages which had been marked down on the first scrutiny as having carried-over had not done so, or there may have been an error in charting their positions; while (2) some marked doubtful might have been found on careful inquiry to have actually carried-over, and the rectification of such errors might lead to some modification in the position of the curve

The re-examination of the records involved a very careful scrutiny, especially bearing in mind the considerations referred to in Appendix C, and in many cases a visit being paid to the village to ascertain, if possible, any circumstances which might lead to a more correct judgment in the matter

For example, Mishrikot, Taluka Kalghatgi, after the preliminary survey, had been marked down, in view of its following record of plague-incidence, as a place that had carried-over —

TABLE IV

Nov	Dec	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct
X	0	X	X	X	X	0	0	0	X	X	X

But, upon examination, the village registers showed that there had been *no* infection in November, and none in April, while the August infection, it was ascertained, came from a neighbouring village Ugginken.

All the details of such special inquiries cannot be given here but some typical examples are included in Appendix B.

An interesting fact emerging from these investigations was that no definite evidence of a 'doubtful' place having carried-over could be obtained, and as a nett result there was no shifting of the curve on the chart so as to diminish the area representing carrying-over.

The chart having been prepared in this way enabled the construction of the following formula showing the dates after which places of various populations have a chance of carrying-over —

TABLE V

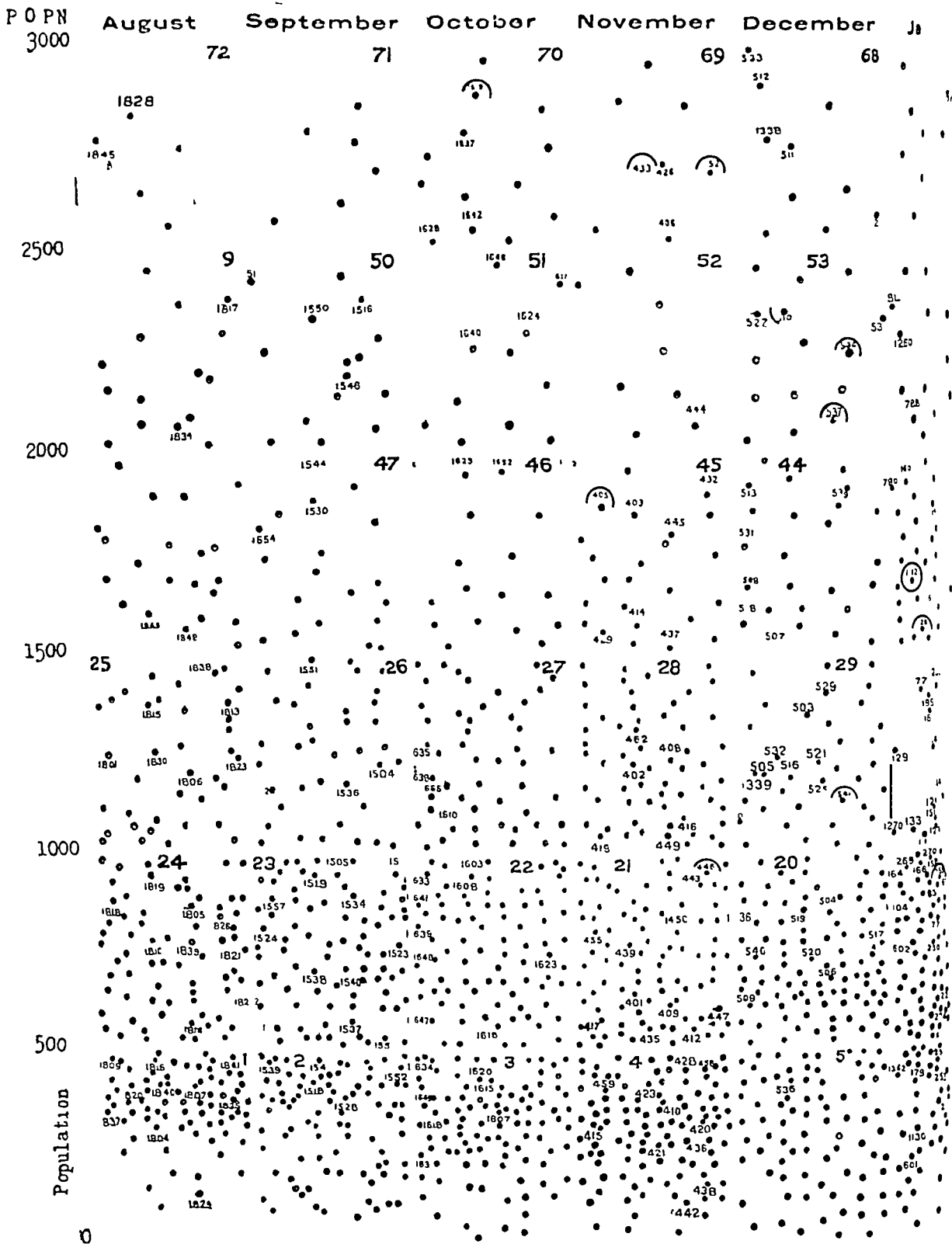
Places with a population of	Infected after	
1— 50	May	1
51— 500	March	1
501—1 000	January	22
1,001—1,500	January	1
1,501—2,000	December	15
2,001—2,500	December	8
2,501—3 000	December	1

} have a chance of carrying over

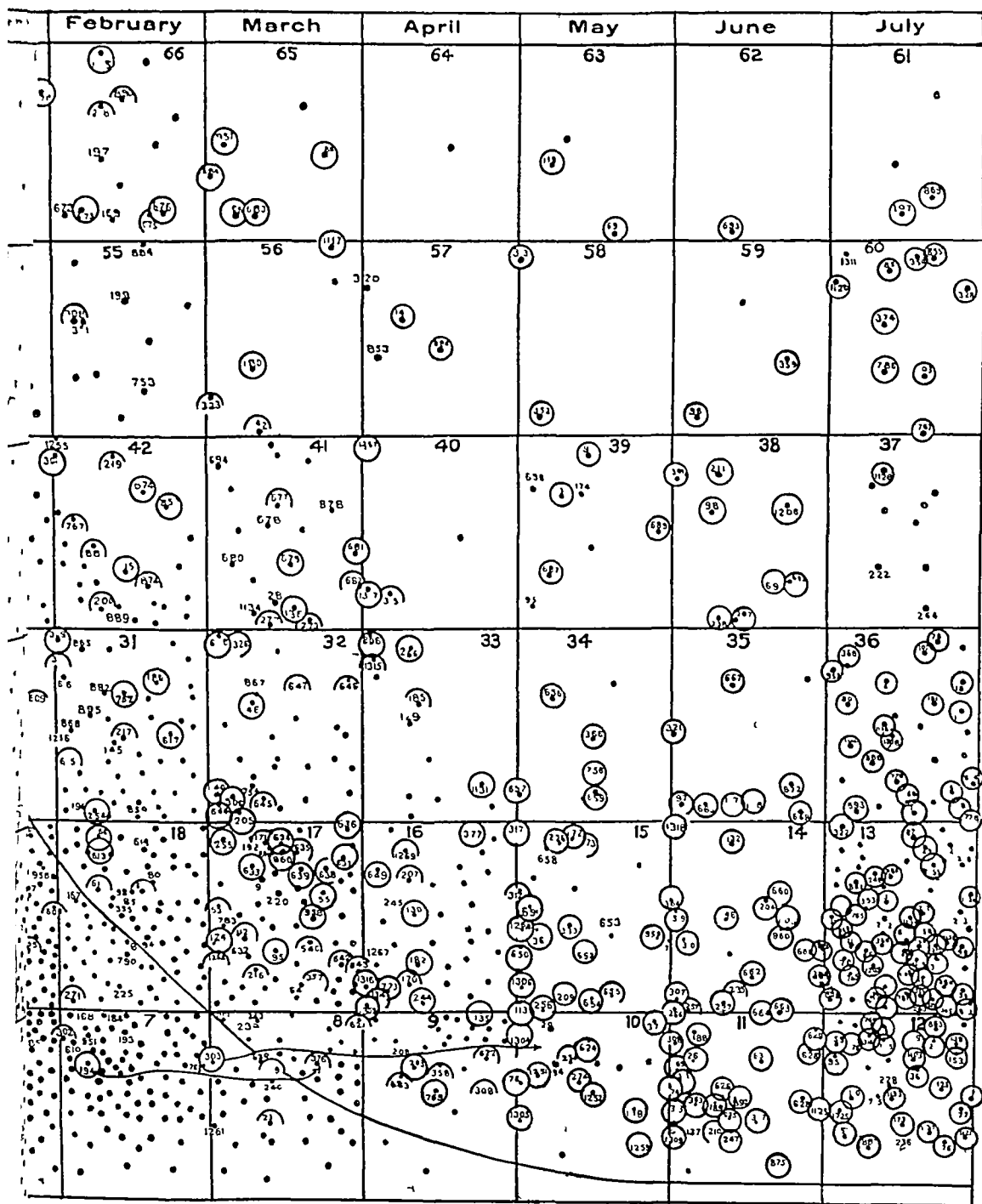
In concluding this section it must be reiterated that the principles and methods employed were exactly those of Kunhardt and Chitre, consultation with whom the writer was happy to enjoy, and the results in the respective regions surveyed may be taken to be strictly comparable.

B Probability of importation

Theoretically, the later an infection of a village, the less the probability within any fixed period of its subsequent reinfection by a fresh importation, the reason being that the prior infection sets up a defensive mechanism in both the population and the rats, for instance the population disperses, and the rats also flee from their own dead, and this mechanism renders the soil less favourable for the reception of fresh seed. Moreover, the later the prior infection the later normal conditions return,—people who have fled come back to their homes, the rats multiply to make good their losses, and so on,—and *the later* the re-establishment of normal conditions, the less is the chance of reinfection taking root in a place within any given period of time.



The abscissæ represent months of primary infection from August to July, of villages. The ordinates the population. When a village became reinfected in the succeeding plague year the dot representing it on the chart was difficult, the number was doubtful, it was half ringed, if the place seemed certain to have been reinfected. Finally the villages which seemed to be eccentric were investigated and if necessary their position altered.



if the village in grades of 500 base line being zero

numbered, and its records scrutinized with a view to determining if it had 'carried over' If the interpretation
 er, its number was completely ringed
 (Block VII)

Actually the following are figures of rates of presumed importation obtained from Chart 1 and they exhibit that anticipated decline —

TABLE VI
Giving calculated importation-rates into villages —

Infected in	Populations		
	1—500	500—1,000	1,000—1,500
August-September	10.7	13.6	14.0
October-November	8.2	12.0	9.7
December-January	5.1	11.8	

The chart also shows fairly clearly outside the carrying-over 'area' that *the bigger a place is*, given its infection at any time the greater the chance of its reinfection by importation. It is not difficult to understand this: not only must the chances of human plague be proportionate to the numbers of susceptible people, but the bigger a place the more road and rail communications it has and these bring an increased liability to infection. Further, the larger a place is the more is the chance that it be a centre of trade, or grain-centre, or a bazaar-town, and as a final reason the greater chance that when the primary infection died out it will have left a larger proportion of susceptible people.

It may incidentally be noted that even in comparatively small villages the importation rate is high, the chance being about one in five. What then must be the odds in big towns?

C Phases of the 'plague-year'

In the official Bombay Government forms kept for the record of plague-incidence (see Appendix A), the 'off-season' was marked as from April to June inclusive. On the other hand in the *Reports on Plague Investigation in India* published by the Indian Plague Commission (1910) charts are given showing that the average number of fleas per rat (see Chart 2), as well as human plague-incidence increases from the middle of May.

Now, as the off-season should rightly be considered to be at an end when the correlated factors favouring plague begin to wax in power, then in Belgaum, where one factor, the flea-rate, does so from the middle of May, this time and not the end of June should be taken to be the end of the off-season. That it is so is further supported by the following two tables showing (1) the number of villages with populations up to 3,000 which became infected month by month during the period under review, and (2) the monthly plague death-rate over the same period.

TABLE VII

The number of villages of populations up to 3 000 which became infected month by month, 1914-1920

Months	Number of villages infected	Months	Number of villages infected
January	440	July	149
February	260	August	227
March	172	September	256
April	86	October	291
May	60	November	277
June	68	December	242

TABLE VIII

The total monthly deaths from plague, 1914-1920

Months	Number of deaths	Months	Number of deaths
January	10,770	July	3,362
February	8,911	August	6,097
March	6,269	September	8,756
April	2,736	October	8,599
May	1,423	November	9,021
June	1,511	December	8,117

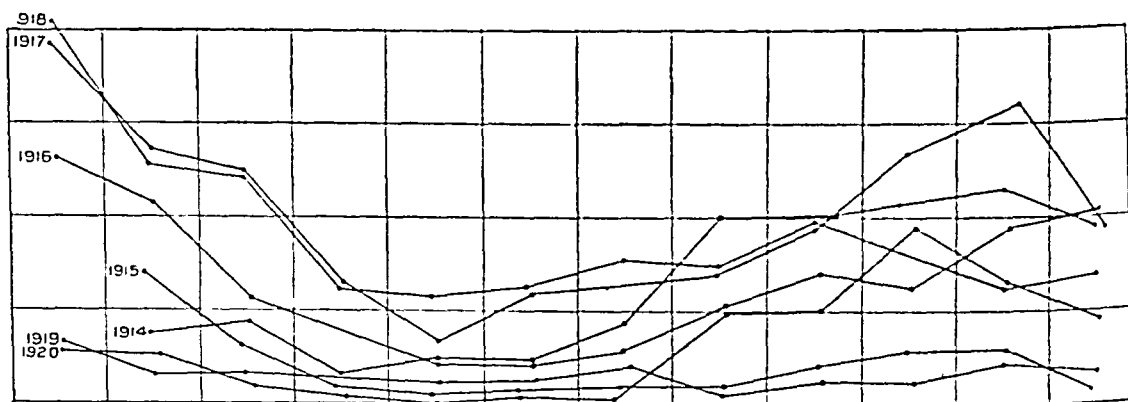
These tables show that the case-incidence, and particularly the village-infections, increase slightly after May and go on increasing till October they then decline slightly, but rise again in January to a very high level. After January until May the village-infections and the plague-death incidence decline gradually, thus completing the cycle.

The tables and Chart 2 then both prove that the off-season terminates at the middle of May and not at the end of June*

* Special efforts in sanitation should nevertheless be made all through May and June

cheopsis-rate and for this there are no differential data available for the two zones, except that Cragg has reported that at Athni, well within the *desh* country the flea were all *cheopsis* and that at Belgaum lying between the *desh* and *malad* the *cheopsis* rate increases up to September *ashta* being absent in both places. The difference seen in the plague-incidence in the two zones is therefore more probably correlated with some differences in the agricultural industries of the people for instance, the *desh* being largely cotton-growing has to import more grain

CHART 3



Showing the different plague incidence in the *malad* and cotton *desh*

The close correspondence of the plots for most of the year, which probably is not accidental, is very suggestive that the difference from August to December is a true one and not a sampling error

SUMMARY AND CONCLUSIONS

The object of the analysis here made was, it may be reiterated to discover, as far as possible, the circumstances in the Belgaum and Dharwar Districts under which 'carrying-over' takes place, and to shed light on cognate matters all of which were of practical importance in the prevention of plague in these districts

The carrying-over problem was investigated by charting the available data, viz the case-records of infected villages, and the analysis, while confirming the importance of the factors of population and month of first infection of a village, has provided a new formula for the conditions of population and month of first infection under which villages in these districts have any chance of carrying-over

With regard to other matters the probability of reimportation into a previously-placed has been shown to decrease *pari passu* with the lateness of the primary while on the other hand, *ceteris paribus*, the larger an infected village the chance of reimportation in the following plague season

all towns of 2,000 to 3,000 population, infected early (August) in the (May-April), the reimportation-rate next year was about 20 per cent in larger towns, and without the inhibitory influences raised by a must be very high indeed*

* The rate was not calculated

The phases of the plague-year have been shown to be as follows the 'epizootic season' may be taken to start at the middle of May and to reach its zenith by the middle of the following January. The *off-season* then commences and continues to the middle of May. It is certainly most important to conduct anti-plague operations very rigorously from the onset of the off-season.

A well-marked secondary rise of human plague-incidence occurs in January when the annual epidemic reaches its zenith, but the flea-rate is then near its *minimum*. An explanation for this may be that the transport of the harvest at this season affords rats and fleas the greatest possible opportunity to spread the disease in spite of the low flea prevalence.

There is a distinct difference in plague-incidence between the eastern dry zone (or cotton *desh*) and the more humid western *malad* (the paddy-growing zone). Probably the differences seen are bound up with some of the industrial conditions of the people, e.g., the growth of the staple crops, or they may be merely reflections of the varying temperatures or other physical condition, or they may be due to the *desh* being more largely importers of grain.

From the analysis it will have been seen, to revert to the subject of carrying-over, that one particularly striking point has emerged, viz., that in these districts carrying-over occurs in places of a very much smaller size and of an earlier month of infection than was Kunhardt's experience in the districts around Poona. In fact, except in a very favourable year, the number of places which would have to be marked down as possibly 'carrying-over' would be comparatively large.

Comparing the carrying-over formula constructed by Kunhardt for the Poona District with that resulting from this analysis it seems that there is a true difference between plague-incidence in that area on the one hand, and Belgaum and Dharwar Districts some 200 to 400 miles south on the other (see Chart 1). Kunhardt has kindly shown me an unpublished paper by himself in which he points out that in an equable and humid climate such as in Madras Presidency during the non-epidemic season plague smoulders on* and there is no well-marked epidemic season whereas in the arid Punjab the epidemic season is very well marked and in the off-season there is but little manifestation of the embers of the fire burning beneath the surface†. It is a very suggestive fact that Greenwood working with data from North India concluded that except for 'large villages' the distribution of 'multiple infections' was fairly random, in other words, carrying-over was not very evident in small villages.

If it be only climatic conditions that produce these extreme results, it is quite possible that the Belgaum and Dharwar Districts with their more humid climate would approximate to the Madras state of affairs, and the Poona Districts with their drier climate more to that in the Punjab. And that too may be the reason for the different plague-incidence that one sees in the *malad* and *desh* of the Belgaum and Dharwar Districts.

From a practical point of view these conclusions would indicate that in the more northern arid zones it might be possible easily to stamp out plague by operations based on the same principles as the Belgaum and Dharwar Districts Plague

* In other words sporadic human cases are seen.

† This simile which is often used for carrying over plague seems inapt there is a burning raging fire beneath and sometimes but a glow comes to the surface.

Operations, whereas it might not be at all possible in the more southern regions. At any rate in the Belgaum and Dharwar Districts the proposition might be a far bigger one than originally contemplated, while farther south the scheme might be quite impossible.

If the incidence of plague varies between the *malad* and *desh* of the Southern Maratha country and between this and the Deccan, only two to four hundred miles away, in no region would one be able to postulate from experience in other parts of the country what conditions are obtaining in it.

Therefore, to conclude, in whatever part of the country for which operations may be designed, *it is essential that there be made a preliminary survey of the plague records*.

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APPENDICES

- A FORM IN WHICH PLAGUE RECORDS WERE KEPT IN THE BOMBAY PRESIDENCY
- B INQUIRIES REGARDING THE EPIDEMICS IN CERTAIN VILLAGES, WITH A VIEW TO DETERMINING WHETHER THEY 'CARRIED OVER'
- C INTERPRETATION OF RECORDS
- D INDEX TO THE NUMBERED PLACES OF CHART 1

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APPENDIX A.

Plague. _____ Chart

_____ Registration District

_____ Collectorate

_____ Taluka

Population	Name of town or village	Position												REMARKS		
		December 1919	January 1920	February	March	April	May	June	July	August	September	October	November	December	Date of outbreak	
2,300	Khanapur					1	1			1	1	1	1		17th	Carried over
21,009	Gadag		1	1											3rd	Did not carry over
3,743	Masur		1								1		1		5th/21st	Imported infection in September

Form in which the plague records were kept in the Director of Public Health's Offices, Bombay Presidency, with hypothetical examples The numerators of fractions represent the number of surviving cases, the denominators the deaths

APPENDIX B

Notes on the plague-incidence in certain villages

The following notes relate to places which were the subject of special inquiry, with the conclusions reached regarding them having carried-over or not, as judged by all the available criteria —

Kasambi, Taluka Kod No 1303

Infection in 1915 as follows —

Jan	Feb	Mar	Apr	May	June	July	Aug	Sept
		1 ⁶		11				

The infection in May had been marked down as 'carried-over' but was thought to be possibly a reimportation from Hedigond about 3 miles away or from Chinnikatti some 4 miles away, both of which were infected at that time — in fact the whole of this part of the taluka was heavily infected. The people of those villages have intercourse through their common bazaar-village, Kagmelli, lying midway between them, and therefore this Kasambi epidemic has been taken to have been a case of reimportation in May carrying-over into June.

Asoge, Taluka Khanapur, population 360 Block VII, No 194

The infection-incidence appeared in the official records to have been a normal carrying-over from February into June as follows —

Feb	Mar	Apr	May	June	July	Aug	Sept
1	6	7	8	3	2		1

but upon inquiry it was ascertained that the outbreak of the infection had been in March (on the 23rd), not in February, and the place was accordingly marked in the chart as carrying-over only from March. It may be added that there was no evidence of a superposed* reimportation here, although it was a place of pilgrimage. People of almost all classes of the district visited it on the day of Mahasivratri in the month of March every year, when the attendance might be 2,000.

Nellbid, Taluka Hangal Block VIII, No 231

This place was marked down at first as an instance of carrying-over, on the strength of the following register of infection in 1916 —

Mar	Apr	May	June	July	Aug	Sept	Oct	Nov
4	1		8	4	3			

* See p 50 of Appendix C

However, upon inquiry, it appeared likely to have derived its June infection from Kalvekallpur one mile away, or from Marianbid, a little further, both of which had previously become infected from their common bazaar-village Adur. One may add here that in the meantime Adur had lost its infection and that later on Nellbid apparently reinfected it, an interesting instance of a bazaar-village setting up infection in a cycle of villages and becoming ultimately reinfected from one of them.

Asoge, Taluka Khanapur Block VIII, No 376

Infection in March 1920 as follows —

Mar	Apr	May	June
1			-

Asoge again probably owed its primary infection (*see above*) to the fact of its holding its fair in March. However, the infection in June was, it seemed, almost certainly derived from Khanapur, only 2 miles away, which was heavily infected at the time.

Bachinkeri, Taluka Sampgaon, population 373 No 109

Infection in March 1915 as follows —

Mar	Apr	May	June	July	August
1	6		2	2	3

There appeared to be no place whence in June it could have suffered reimportation, and it was marked as having carried-over.

Dadarkop, Taluka Parasgad Block VIII, No 621

Infection in March 1918, as follows —

Mar	Apr	May	June	July	Aug	Sept
-				-	1	1 $\frac{3}{4}$

Incidence like this was unlike the 'standard' instances of carrying-over. It seemed to have become reinfected from Balur in Navalgund, and was not carried-over.

Kardikop, Taluka Hubli Block XVIII, No 271

Infection in February 1916 as follows —

Feb	Mar	Apr	May	June	July	Aug
$\frac{1}{2}$	$\frac{1}{2}$				$\frac{1}{16}$	etc

It was probably reinfected from Palikop 3 miles away, and was not then a carrying-over village

Hebbal, Taluka Khanapur Block XVIII, No 611

Infection in February 1917 as follows —

Feb	Mar	Apr	May	June	July	Aug
$\frac{1}{2}$	$\frac{1}{6}$			$\frac{1}{16}$	$\frac{1}{8}$	

This appeared to have carried-over, but as it lay within a few miles of Khanapur Bazaar on the main trunk road, it was the object of special inquiry however, no information on the point could be obtained

Chikalgad, Taluka Hukeri Block XVIII, No 612

Infection in February 1917 as follows —

Mar	Apr	May	June	July	Aug	Sep	Oct	Nov	Dec
$\frac{1}{6}$	$\frac{1}{6}$	$\frac{1}{6}$	$\frac{1}{6}$	$\frac{1}{6}$	$\frac{1}{6}$	$\frac{1}{6}$	$\frac{1}{10}$	$\frac{1}{6}$	$\frac{1}{6}$

The case in August might have been a manifestation of endemic plague due to carrying-over, but was found to have been that of a Brahman woman visiting the village from Thanahatargi which was then plague-stricken The place was consequently marked down as not having carried-over

Gumgol, Taluka Navalgund Block XIX, No 265

Infection in January 1916

This infection had carried on until April when it ceased, but had recurred in August This might very well have been the case of carrying-over, but upon investigation it was found that in all probability it was a case of reimportation from Morab which lies only a furlong away

Chikkur, Taluka Kod Block XXI, No 446

Infection in November 1916

This place had apparently carried-over but it lay on a track between Ranibenur and Honnali on which another village Tuminkatti was situated. Tuminkatti had become infected in January and Kappelur, its bazaar-town, in April, and apparently this place had been instrumental in reinfesting Chikkur during the plague-season

Sherwad, Taluka Hubli Block XXIX, No 211

Infection in 1919

The records of plague in this place had shown infection from November to February, then in July, and again in November and December. But upon inquiry it was found that the infection reported in July had been an error and there could therefore be no doubt that this place did not carry-over

Kochari, Taluka Hukeri Block XXX, No 609

Infection in 1917

This apparently was as certainly as could be a case of carrying-over, but as it lay near Hukeri and Shankeswar, both heavily infected at the time, it might have been an example of reimportation simulating carrying-over

Thanehatagi, Taluka Hukeri Block XXXI, No 615

Infection in 1917

In the tables the incidence was apparently an example of carrying-over, but on inquiry the village registers showed no infection in 1917

Dhupdal, Taluka Gokak

Infection in March 1916

The incidence of the infection had been in March and April, then nil until July during this immune period no history of rats dying could be elicited, and the place was therefore considered not to have carried-over

Ganjgatti, Taluka Kalghatgi No 617

Infection in 1919

This place did not present any normal type of incidence, but on the other hand there could be discovered no source, nor any history, of reimportation. The matter had to be left in doubt

Janwad, Taluka Chikodi Block XLIII, No 120

Infection in 1915

The incidence of infection missed out April to July and in August the place was probably reinfected from Sadale one mile away which was heavily infected at the time

Sidnur, Taluka *Kod* No 162

Infection in 1915

This place presented an incidence of infection a little atypical and possibly suffered reimportation from Annur or Kerewadi, both of which were quite close to it

Adur, Taluka *Hangal* No 365

Infection in 1920

As mentioned above in connection with Nellibid, Adur was probably reinfected from this village, which was one in a cycle which the primary cases in Adur had infected

Devalpur, Taluka *Sampgaon* No 780

Infection in 1917

This place seemed as typical a case of carrying-over as one could find, but the infection at the outset in January was perhaps due to importation of human-plague direct and not due to any epizootic, in which case it seemed correct to label the infection as carrying-over only from March, and not from January

Amangi, Taluka *Hukeri* Block XIV, No 405

Infection in 1916

This place had shown infection till April 1917, and apparently derived its infection in the plague-season by reimportation from Hukeri or Shankeswar Bazzars

Kalghatgi, Taluka *Kalghatgi*

Infection in 1915

This infection had continued into 1916, but then missed out May, June and July, and its later infection had been probably derived from Bommingatti, 7 miles, or Devikop, 4 miles away, for both of which villages it was the bazaar-town

Solapur, Taluka *Hukeri* Block LIV, No 1312

Infection in 1915

This place had suffered infection from January to June continuously, which apparently had exhausted its potentialities for further cases because the epidemic did not continue after it had carried-over into June, and moreover the place did not suffer reimportation from Shankeswar, a mile away, which was heavily infected during the plague-season

Sampgaon, Taluka *Sampgaon* Block LXVIII, No 1319

Infection in October 1916

This infection in October in the register had consisted of 2 cases only with nothing further till January, after which it had carried-over

But on inquiry it was found that no case had really occurred in October, there had been one on December 1st in a man who was reported not to have been away from Sampgaon except to work in the jungle, and after this the infection had started on December 28th and carried-over. The case on December 1st had probably started the epizootic.

APPENDIX C

Considerations applied to the interpretation of correctly-registered epidemic records

(1) In order to justify a conclusion that a place had carried-over some manifestation during the off-season of plague was required

(2) If tables of what may be called standard epidemics were drawn up for places (a) of a given grade of population to show the type of plague-incidence when the onset of infection occurred in the several months of the year, and (b) of different grades of population primarily infected in any given month, and it was found that the incidence of plague in any place conformed to these standards, then that place was taken to have carried-over. If, however, the incidence records were slightly aberrant from the type, an investigation might be made, after which, if a positive or negative decision could not be arrived at, the place was classed as 'doubtful' (See Standard Tables at end of the Appendix)

(3) The possibility of reinfection being due to reimportation from an adjacent plague-focus was borne in mind. On the other hand in nearly every case of undoubted carrying-over there was some adjacent source of possible reimportation.

(4) Trade, rail, and road communications with infected foci were factors which went to determine likelihood of reimportation. Places of pilgrimage or bazaar-towns being infected were factors predisposing to the spread of plague to places with which they held traffic.

(5) The street and house incidence of the disease was considered, this, if confined to one house, gave rise to a suspicion of importation, whereas when several cases in diverse localities occurred carrying-over was suggested.

(6) A history of a rat-epizootic contingent on an epidemic was important evidence of carrying-over, but it was not often obtainable.

(7) An explanation of the course of certain village epidemics will show how difficult it sometimes is to interpret them.

When a place becomes first infected, a state of affairs is set up which tends to inhibit the continuation of an epidemic and the longer this lasts the more pronounced is the development of that state, and when the infection has come to an end a normal condition is gradually re-established. The chance of an importation grafted upon that primary epidemic is governed by those considerations. Even when the factors which are inhibitory to the continuance of the epidemic have developed to the pitch that the epidemic ceases, importation is not precluded, for no epidemic quite exhausts the supply of susceptible human subjects, but if the infection of these susceptibles has not come about 'endemically', it is far less likely to occur with the infected population of another village as the source.

However, when scrutinizing incidence records, it is sometimes easy to see that *part of the incidence has been due to an importation engrafted on the original epidemic*. For example, see the course of infection in the following —

Mallapur K Ankalgi, Taluka Gohak, population 642

Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
	13th							9th			
	1	2						1	4	1	1

The second outbreak on the 9th September was, as far as one could judge, a reimportation

But supposing this reimportation had been superposed on the original infection in April instead of in September, the following would have been the result —

Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec	Jan
13th											
1	2	1	1	1	1						

and monthly incidence being continuous would have had the appearance of carrying-over from February 13th, a conclusion which would have been erroneous

An indication of the occurrence can sometimes be noticed in plague-incidence tables, for instance, in *Shankeswar, Taluka Hukeri, population 6,717, infected 18-1-17* —

	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
1917	21	33	36	36	18	1	29	36	17	1	51	23
1918	12	10	9									

From January to October 1917, the incidence can be described as normal, there being a gradual crescendo at first, and then a decline from April (in the off-season) until a rise rightly begins at the opening of the plague-season (in July). The epidemic, however, begins to fall away in September, even though conditions are then becoming more favourable for the spread of the disease, *when there is a sudden recrudescence* in November which carries on the epidemic to March

Now this recrudescence was probably the result of reimportation from Kochari, for compare —

Hukeri, population 5,258, infected 30-1-17, that is, a town of about the same population as Shankeswar and infected primarily at the same date. In *Hukeri* apparently no such reimportation took place

	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
1917	$\frac{1}{2}$	2	~	$\frac{13}{10}$	$\frac{17}{10}$	$\frac{14}{12}$	58	$\frac{100}{185}$	44	11	8	

Places like Shankeswar therefore with their apparent carried-over plague record which was, it may be concluded, due to a reimportation superposed on an original infection, must have led in the analysis to many misinterpretations and swollen the number of places determined as carried-over consequently the graphic method employed above for determining the circumstances under which carrying-over takes place must have been prejudiced in the direction of increasing the area representing carrying-over

Thus, it was concluded that number 1303 in Block 8 on Chart 1 representing Kasambi, in Kod Taluka, carried-over. In this place the plague-incidence in 1915 was as follows —

Jan	Feb	Mar	Apr	May	June	July	Aug
		$\frac{15}{10}$		$\frac{11}{10}$			

But on inquiry it was ascertained that the infection in May was probably due to reimportation from Hedgond, or Chinnikatti, respectively 3 and 4 miles away, which were both infected in May

As stated, such conclusions must have considerably influenced the position of the curve on Chart 1

APPENDIX C—*contd*

Tables showing plague-incidence in 'Standard' epidemics

Onset of infection in	In places of population	Place	Taluka	First infection	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
August	1-500	Dhupadhalli	Kod	28-8-20	1	13	1	1				
	500-1,000	Mulali	Bank	15-8-20	21	33	1					
	1,000-1,500	Mangundi	Har	8-8-20	16	11	2	1				
	1,500-2,000	Amargol	Hub	31-8-17	1	2	13	2				
	2,000-2,500	Hailapur	Gadag	31-8-17	3	12	14	42	5			
	2,500-3,000	Ankalgi	Gok	-8-15	46	71	33	23				
SEPTEMBER	1-500	Sidapur	Ath	22-9-17		2	1	2	5	13		
	500-1,000	Avredti	Sam	19-9-16		11	0	10	1			
	1,000-1,500	Dhumwad	Kal	21-9-21		13	7	7	20	15	1	1
	1,500-2,000	Septisagar	Ath	18-9-17		21	44	14	2			
	2,000-2,500	Kotalgi	Ath	18-9-16		40	11	2	1			
	2,500-3,000	Devihosur	Karj	29-9-17		1	12	16	17	10	4	

APPENDIX C—contd

Onset of infection in	In places of population	Place	Taluka	First infection	Dec	Jan	Feb	Mar	Apr	May	June
DECEMBER	1-500	Budalmukha	Chik	28-12-18	4	4	4	4			
	500-1,000	Sangrasakop	Par	13-12-18	4	1	1 ²	"			
	1,000-1,500	Bhindigen	Bel	12-12-16	1	4	15	18	1		
	1,500-2,000	Karekatth	Par	22-12-17	4	16	10	4	1		
	2,000-2,500	Kalghath	Kal	30-12-17	4	10	14	15			
	2,500-3,000	Nesargi	Sam	10-12-16	13	13	20	17	1		
JANUARY	1-500	Lolsur	Gok	1-1-16	4	4	10	4			
	500-1,000	Badas	Bel	21-1-17	4	4	17	14	3		
	1,000-1,500	Kamatnur	Huk	9-8-17	11	11	10	11	1		
	1,500-2,000	Halshi	Khan	1-1-16	10	10	14	10	3	2	
	2,000-2,500	Hebtal	Huk	19-1-17	1	1	10	14	14	3	
	2,500-3,000	Yezgatti	Mur	1-1-16	1	1	4	4	1		

APPENDIX C—*contd.*

Onset of infection	In places of population	Place	Taluka	First infection	Feb	Mar	Apr	May	June	July	Aug	Sept
FEBRUARY	1—500	Margankop	Sam	28-2-17	1	10	37	17				..
	500—1,000	Manoli	Khan	9-2-17	7	12	13	6	9	2		
	1,000—1,500	Kochani	Huk	1-2-16	4	18	9	4				
	1,500—2,000	Adi	Chik	13-2-16	7	15	11	10	37	10		
	2,000—2,500	Narendga	Dhar	1-2-14	10	4			1	3	4	
	2,500—3,000	Ankhi	Chik	1-2-15	9	5	16	14	13	15		
		Alladkatti	Kal	16-3-16		14	11	1				
	500—1,000	Hedigond	Kod	18-3-16		1	17	1		7	8	
	1,000—1,500	Hulkatti	Samp	21-3-16		1	12	19	14	11	1	
	1,500—2,000	Shundur	Chik	16-3-19		9	4	2		19	13	15
MARCH	2,000—2,500	Hebbal	Huk	24-3-17		1	7	7	7	17	11	23
	2,500—3,000	Negunhal	Sam	1-3-17		19	14	19	1	11	1	1

APPENDIX C—concl'd

Onset of infection in	In places of population	Place	Taluka	First infection	June	July	Aug	Sept	Oct	Nov
JUNE	1—500	Hindasgen	Kal	1-6-20	$\frac{1}{2}$	$\frac{1}{17}$	$\frac{19}{17}$	7	2	-
	500—1,000	Nalknur	Nav	19-6-20	$\frac{1}{1}$	2	5	$\frac{1}{7}$	1	-
	1,000—1,500	Bidi	Khon	27-6-18	1	-	$\frac{1}{7}$	$\frac{1}{1}$	$\frac{1}{4}$	-
	1,500—2,000	Buzruk Arlikati	Hubli	22-6-19	$\frac{2}{1}$	3	27	$\frac{15}{15}$	$\frac{7}{7}$	-
	2,000—2,500	Tadas	Bank	21-6-20	$\frac{1}{2}$	$\frac{3}{11}$	$\frac{1}{3}$	1	$\frac{19}{11}$	-
	2,500—3,000	Masur	Kod	13-6-18	$\frac{1}{11}$	$\frac{19}{17}$	$\frac{1}{11}$	$\frac{12}{11}$	$\frac{13}{13}$	-
JULY	1—500	Chikmanoli	Khan	6-7-17	$\frac{1}{1}$	$\frac{1}{1}$	7	$\frac{1}{1}$	$\frac{1}{1}$	-
	500—1,000	Balambid	Han	8-7-17	$\frac{1}{1}$	$\frac{1}{1}$	$\frac{20}{20}$	$\frac{12}{12}$	$\frac{19}{19}$	-
	1,000—1,500	Anjur	Ath	18-7-17	$\frac{1}{1}$	$\frac{1}{1}$	$\frac{1}{1}$	$\frac{1}{1}$	$\frac{19}{19}$	-
	1,500—2,000	Mugad	Dhar	22-7-16	$\frac{1}{4}$	$\frac{1}{4}$	17	$\frac{20}{20}$	$\frac{19}{19}$	-
	2,000—2,500	Khanapur	Khan	12-7-17	$\frac{1}{1}$	$\frac{1}{1}$	$\frac{19}{19}$	$\frac{18}{18}$	$\frac{11}{11}$	-
	2,500—3,000	Lakkundi	Gadag	11-7-17	$\frac{1}{1}$	$\frac{1}{1}$	23	$\frac{17}{17}$	1	-

APPENDIX D

*Index of Registered Numbers of all Places of 1 to 3,000 population,
shown on Chart 1*

Registered number	Place	Taluka	Registered number	Place	Taluka
<i>January to July 1915</i>			<i>January to July 1915—concl'd</i>		
77	Ankalgi	Belgaum	160	Havasbhavi	Kod
80	Nandihalli	"	161	Aladkeri	"
83	Devlatti	Khanapur	162	Sidenhur (Old)	"
85	Itagi	"	164	Dudihalli	"
88	Harugeri	Athani	166	Hirehallur	"
91	Adhalli	"	167	Guddadkeri	"
93	Jugul	"	168	Nittur	"
94	Yelbarhatti	"	169	Masur	"
95	Hidkai	"	170	Annur	"
96	Nandgaon	"	171	Chikyadachi	"
97	Hallibai	"	172	Chikkabur	"
98	Sapsagar	"	173	Kervadi	"
99	Tangadi	"	174	Kadur	"
101	Khamalapur	"	175	Tadas	"
102	Nandeswar	"	176	Hirehalli	"
103	Mahiswadgi	"	177	Ablur	"
104	Sankarhatti	"	178	Hirebudihal	"
105	Mudalgi	Gokai	179	Hoskatti	Bankapur
108	Kumkeri	"	180	Tadas	"
109	Bachinkeri	Sampgaon	181	Mattikatti M Karadgi	"
111	Hitani	Hukeri	182	Taramamali	Navalgund
114	Belambi	"	184	Hullatti	Hangal
116	Karagali	"	185	Herur	"
117	Borgai	"	187	Kappasikop	"
118	Daddi	"	188	Tarihal	Kalghatigi
120	Jenawad	Chikodi	189	Bulnakan hulikatti	"
121	Rajapur	"	<i>January to July 1916</i>		
123	Mamdapur	"	190	Gunjagi	Belgaum
125	Ankali	"	191	Nandihalli	"
128	Nandi	"	192	Halsli	Khanapur
129	Belkud	"	193	Kamasinkop	"
130	Umarani	"	194	Asoge	"
131	Mankapur	"	195	Hirehattihoh	"
133	Kotur	Karjgi	196	Jambagi	Athani
136	Mallur	"	197	Kagwad	"
137	Bechinkatti	"	198	Hulikatti	Parisgad
138	Timmapur	"	199	Gurhosur	"
139	Didgur	"	200	Yakkundi	"
142	Asundi	Ranebenur	201	Badli	"
145	Benkundi	"	204	Vaklund	Sampgaon
146	Kuppelur	"	205	Hulikatti	"
147	Kadarmandalgi	"	206	Belwadi	"
149	Rangoundanahalli	"	207	Nichanki	"
150	Nangenhalli	"	208	Basapur	"
151	Kallapur	"	209	Kotbagi	"
153	Agasanahalli	"	210	Giryal	"
156	Varur	Hubli			
158	Yettnihalli	Kod			

APPENDIX D—contd

Registered number	Place	Taluka	Registered number	Place	Taluka
<i>January to July 1916—contd</i>			<i>January to July 1916—concld</i>		
211	Devaipur	Sampgaon	270	Gammankatti	Hubli
212	Chivatgundi	"	271	Kardikop	"
213	Gavankop	"	272	Mantur	"
214	Mallapur	"	273	Amergol	"
216	Kamankatti	Gokak	274	Palikop	"
217	Dhupdal	"	<i>January to July 1911</i>		
218	Manoh	Hubli	301	Kanbargi	Belgaum
219	Yeddur	Chikodi	302	Kimiyabailur	"
220	Sidnal	"	303	Karle	"
222	Shendur	"	304	Belgundi	"
223	Talhalli	Karjgi	305	Waghawade	"
224	Melhgatti	"	306	Topinkatti	Khanapur
225	Yellapur	"	307	Kasaba Nandgad	"
226	Kittur	"	308	Ugur	Athani
227	Bharadi	"	309	Tigdoli	Sampgaon
228	Kamalbangadi	"	311	Hulloli	Hukeri
229	Aleswar	Hangal	312	Chukalgud	"
230	Belgalpeth	"	313	Khanapur	"
231	Nellibid	"	315	Nandi	Chikodi
233	Maianbid	"	316	Gariwad	"
234	Hulliguddi	"	317	Turkewadi	Chandgad Mahal
235	Sankrikop	"	320	Dergiri	Karjgi
236	Jalnakankop	"	321	Ganjgatti	Kalghatgi
238	Airani	Ranchenur	322	Arleswar	Hangal
239	Ibrampur	Bankapur	323	Narandgeri	Dharwar
240	Narvaupur	"	324	Kankur	Bankapur
242	Ingalg	"	326	Kudputti	Kod
243	Advisomapur	"	327	Arikatti	"
244	Bad	"	328	Kedur	"
245	Kunnur	"	<i>January to July 1920</i>		
246	Chillarbadni	"	351	Hebour	Hubli
247	Konankeri	"	352	Kod	Kod
248	Devalmalihalli	"	353	Ablur	"
249	Kyalkind	"	354	Hawasbhavi	"
250	Betkerur	Kod	355	Yettinhalli	Bankapur
251	Kachavi	"	357	Mugah	"
252	Kaginelli	"	358	Madli	"
253	Hiremakur	"	359	Tadas	"
254	Makari	"	360	Kunkur	"
255	Hedigond	"	361	Ugginkeri	Kalghatgi
256	Godehikond	"	362	Hindasgeri	"
257	Hoskatti	"	363	Sangedevarkop	"
258	Hullur	"	364	Surshettikop	"
259	Wankkatti	Dharwar	365	Adur	Hangal
261	Rayapur	"	366	Belgalpeth	"
262	Yettingud	"	367	Hombli	"
263	Chandanmatti	"			
264	Mugad	"			
265	Gumgol	Navalgund			
266	Bommigatti	Kalghatgi			
267	Galgi	"			
269	Bhardevarkop	Hubli			

APPENDIX D—contd

Registered number	Place	Taluka	Registered number	Place	Taluka
<i>January to July 1920—concl'd</i>			<i>December 1914 to 1919</i>		
368	Balimbud	Hangal	502	Biselhalli	Ranebenur
369	Sinkulop		503	Aladkeri	Kod
370	Guddadho-halli	Ranebenur	504	Hawargi	Hangal
371	Nigadi	Dharwar	505	Bulgal	"
372	Kudach	Belgaum	506	Melmuri	Karjgi
375	Kangrahi		507	Karur	Ranebenur
376	Aogc	Khanapur	508	Nalvi	Hubli
377	Jamboti	"	509	Devangpeth	"
<i>November 1914 to 1919</i>			510	Kalaghati	Kalghatgi
401	Khairwad	Khanapur	511	Mangsuli	Athani
402	Shed-salwadi	Athani	512	Shindekurbet	Gokak
403	Neerli	Hukeri	513	Amangi	Hukeri
405	Yerguppi	Hubli	514	Kallapur	Parasgad
406	Chikkerur	Kod	516	Bhendigeri	Belgaum
407	Kagehr	Ranebenur	517	Hirehattiholi	Khanapur
409	Mankur	"	518	Devarshighalli	Sampgaon
410	Hiremma-nur	"	519	Bhavihal	"
412	Khairwad	Khanapur	520	Mohare	"
414	Devshighalli	Sampgaon	521	Ambadkatti	"
415	Hanmandhalli	Karjgi	522	Tigadi	"
416	Aldhalli	"	523	Shindekurbet	Gokak
417	Yelwadi	Hangal	525	Hasarur	Karjgi
419	Andalgi	Bankapur	527	Kadarmandalgi	Ranebenur
420	Nirlgi in Karidgi	"	529	Kagnhalli	Kod
421	Katenkeri	Kod	530	Aktangerhal	Gokak
423	Kamsinkop	Khanapur	531	Manpatti	Hukeri
426	Kagwad	Athani	532	Belgalpeth	Hangal
428	Badli	Parasgaol	536	Budalmukha	Chikodi
429	Betsur	"	537	Kodni	"
432	Amangi	Hukeri	538	Kalghati	Kalghatgi
433	Kunnur	Chikodi	539	Halival	Hubli
435	Yellapur	Karjgi	540	Shilwantsomapur	Bankapur
436	Totadyellapur	"	541	Sherewad	Hubli
437	Karur	Ranebenur	<i>January to July 1917 to 1919</i>		
438	Laxmapur	"	601	Tippaikop	Kod
439	Belur	"	602	Kallur	Gadag
442	Konkanhalli	Bankapur	603	Gor-anhal	Parasgad
443	Chikkerur	Kod	604	Tumapur M Amin bhavi	Dharwar
444	Kod	"	605	Kangrahi Khurd	Belgaum
445	Kadur	"	606	Ingali	Hukeri
446	Chikalbur	"	607	Turkewadi	Chandgad
447	Kalharui	Dharwar	608	Attigeri	Bankapur
449	Karamadi	Ron	609	Koohari	Hukeri
452	Benadi	Chikodi	610	Budalmurkha	Chikodi
455	Gondi	Hangal	611	Hebbal	Khanapur
458	Galagnath	Karjgi	612	Chikalgudd	Hukeri
459	Sideevapur	"	613	Manoli	Khanapur
462	Adargunchi	Hubli			

APPENDIX D—contd

Registered number	Place	Taluka	Registered number	Place	Taluka
<i>January to July 1917 to 1919—contd</i>			<i>January to July 1917 to 1919—concl'd</i>		
614	Hadagali S Dambal	Ron	666	Aladgeri	Kod
615	Thanchetgi	Hukeri	667	Bidi	Khanapur
616	Dhundashi	Bankapur	668	Madli	Kod
617	Ganjigatti M Sutgatti	Kalghatgi	669	Manoli	Hukeri
618	Budarkatti	Sampgaon	670	Telsang	Athani
619	Tigadi	"	671	Mundargi	Mundargi
620	Hallikeri	Mundargi	672	Medleri	Ranebenur
621	Dadarkop	Parasgad	673	Yergatti	Murgod
622	Gadikatti	Sampgaon	674	Sangoli	Sampgaon
623	Handigund	Hukeri	675	Tilwalli	Hangal
624	Tumnapur M Adar	Karjgi	676	Medleri	Ranebenur
625	Bengardi	Chandgad	677	Halsi	Khanapur
626	Koulgeri	Dharwar	678	Hallihal	Hubli
627	Samasgi	Hangal	679	Shindur	Chikodi
628	Hawashi	Karjgi	680	Shindur	"
629	Sheshgiri	Hangal	681	Kakamari	Athani
630	Chikhattiholi	Khanapur	682	Kanajgeri	Ron
631	Kembhavi	Sampgaon	683	Masur	Kod
632	Majali	Hukeri	684	Negimhalli	Sampgaon
633	Kotbagi	"	685	Ankalgi	Gokak
634	Badas	Belgaum	686	Aktangerhal	"
635	Madgi	"	687	Chandgad	Chandgad
636	Ukkund	Ranebenur	688	Donir	Mundargi
637	Muddenguddi	Ron	689	Malwanki	Gokak
638	Devlatti	Khanapur	690	Suchal	Murgod
639	Majali	Hukeri	691	Nagvi	Gadag
640	Hirchattihali	Khanapur	692	Goodgeri	Hukeri
641	Benhalli	Chikodi	693	Chikkerur	Kod
642	Pachimal	Sampgaon	694	Surangi	Karjgi
643	Kondagi	Hangal	<i>January to July 1917, 2nd Series</i>		
644	Masangi	Kod	751	Sambagi	Athani
645	Topinkatti	Khanapur	753	Shiratti	"
646	Bidi	"	754	Pathanballi	"
647	Paraswad	"	755	Adahalli	"
648	Hirobidri	Ranebenur	756	Karhalli	"
649	Adkur	Chandgad	757	Bevenur	"
650	Bagadgeri	Kalghatgi	758	Anjur	"
651	Belgundi	Belgaum	759	Halki	Murgod
652	Hanchimal	Hukeri	760	Yarjgi	"
653	Hosur	"	761	Supdala	"
654	Shadguppi	Hangal	762	Shirsangi	Parasgad
655	Amroli	Chandgad	763	Mantogi	Khanapur
656	Meundi	Mundargi	764	Gudikop	"
657	Chikbasur	Kod	765	Chikmandoli	"
658	Mardinaglapur	Sampgaon	766	Halagi	"
659	Vardi	Hangal	767	Kudachi	Belgaum
660	Amargol	Hukeri	768	Hindalg	"
661	Bagadgeri	Kalghatgi	769	Birpankopi	"
662	Karve	Belgaum	770	Chikbagiwadi	"
663	Karvinkop	Khanapur	771	Sundi	"
664	Ramewadi	Hukeri			
665	Dadhi	"			

APPENDIX D—*contd*

Registered number	Place	Taluka	Registered number	Place	Taluka
<i>January to July 1917, 2nd Series—contd</i>			<i>January to July 1917, 2nd Series—concl</i>		
772	Santibastwad	Belgaum	887	Timmanhalli	Ranebenur
773	Patne	Chandgad	888	Asundi	"
774	Albadevi	"	889	Mugad	Dharwar
775	Guddevadi	"	890	Alnawar	"
776	Sonirwadi	"	891	Kerkop	"
777	Unchalli	"	892	Kojalgeri	"
778	Bagigedukarwadi	"	893	Kadnur	Bankapur
779	Turkanshigihalli	Sampgaon	894	Baddikop	Kod
780	Dexlapur	"	895	Shelgambi	"
781	Bedewadi	"	896	Kudupulli	"
782	Sampgaon	"	898	Dadguppi	"
783	Keshavakop	"	951	Havashi	Karjgi
785	Sidsamudra	"	955	Belvagi	"
786	Hunashatti	"	954	Timmapur M Tuttal	"
787	Virapur	"	956	Hampihalli	Nargund
788	Madeval	Gokak	957	Shirshurak	"
789	Aktingerhal	"	958	Benkatti	"
790	Mallapur K	"	959	Belamhatti	"
792	Nandgaon	"	960	Kappli	"
795	Kanthanur	Hukeri			
797	Kasti	"			
798	Hebbal	"			
851	Bugtealur	"			
852	Nerli	"	1101	Dadkop	Parasgad
853	Nisosi	"	1103	Kadagnur	Athani
854	Jintal	"	1104	Nichanki	Sampgaon
855	Kharnapur	"	1105	Chivatgundi	"
859	Mandapur	Chikodi	1106	Shigehatti	Gokak
860	Bhivashi	"	1108	Patgundi	"
861	Bardur	Mundargipetha	1109	Kamaldum	"
862	Shingatalur	"	1110	Hulikotal	Khanapur
863	Shingadrayankeri	"	1111	Adi	"
864	Karalhalli	"	1112	Kanvikarvinkop	Belgaum
865	Hulihatti	Gadag	1113	Kadgatti	"
866	Hindgal	"	1115	Adi	Chikodi
867	Kadadi	"	1117	Hebbal	Hukeri
868	Hatalgeri	"	1118	Madihalli	"
869	Lakkundi	"	1119	Shemapur	Karjgi
870	Beldhalli	"	1120	Devgeri	"
872b	Guchisaga	Navalgund	1121	Shryaka	"
873	Byalal	"	1123	Manoor	"
874	Adur	Hangal	1124	Naiknur	Navalgund
875	Yettinalli	"	1125	Baloor	"
876	Uppunshi	"	1128	Hulkeri	Ron
878	Yerguppi	Hubli	1129	Kalasapur	Gadag
880	Mutagi	Kalghatgi	1130	Tippakop	Kod
881	Hulambi	"	1131	Bilhalli	Kalghatgi
882	Ar amalapur	Ranebenur	1132	Yettinhalli	Kod
884	Halgerifulgaon	"	1133	Dhupadhalli	"
885	Mensinhalli	"	1134	Hirenandihalli	Kalghatgi
886	Gudgudapur	"	1135	Yerinarayanpur	Hubli

APPENDIX D—contd

Registered number.	Place	Taluka	Registered number	Place	Taluka
<i>January to July 1918—concl'd</i>			<i>January to July 1915, 2nd Series—concl'd</i>		
1136	Hirenathi	Hubli	1314	Petha Nandgad	Khanapur
1137	Savur	Bankapur	1315	Kavatkop	Athani
1138	Banur	"	1316	Badachi	"
1139	Havangi	Hangal	1317	Dharanwar	"
1140	Mangundi	Dharwar	1318	Nanur	"
			1319	Shurchatti	Hukeri
<i>January to July 1919</i>			<i>December 1914 to 1919, 2nd Serie</i>		
1201	Timmapur M Adur	Karjgi	1336	Gundkatti	Kod
1203	Madli	Bankapur	1338	Benadi	Chikodi
1204	Kunnur	"	1339	Hanchunal	"
1205	Hiremattur	"			
1206	Buzruk Arkhatta	Hubli			
<i>January to July 1916</i>			<i>November 1914, 2nd Serie</i>		
1251		Khanapur	1450	Janwad	1 Athani
1252		"			
1253		Chikodi			
1254		"			
1255		"			
1256		Hangal			
1257		Ranebenur			
1258		"			
1259		Bankapur			
1260		Kod			
1261		"			
1262		"			
1263		Dharwar			
1264		Kalghatgi			
1265		"			
1266		Hubli			
1267		Nargund			
1268					
1269					
1270					
1271					
<i>January to July 1915, 2nd Series</i>			<i>September 1914 to 1916</i>		
1301	Yerguppi	Hubli	1503	Bhavdevarkop	Hubli
1302	Chimmikalli	Kod	1504	Keshawapur	"
1303	Kasambi	"	1505	Shiswanhalli	Bankapur
1304	Tawargi	"	1511	Khairwad	Khanapur
1305	Guddadamattahalli	Hangal	1512	Itagi	"
1306	Hirekunshi	"	1513	Adi	"
1307	Ujanpur	Kod	1516	Gulhosur	Parasgad
1308	Domnihal	"	1518	Ganikop	Sampgaon
1309	Domanhal	Hangal	1519	Awarowadi	"
1310	Baminhalli	Ranebenur	1522	Bomanhalli	Hangal
1311	Yadur	Chikodi	1523	Hanansagar	"
1312	Solapur	Hukeri	1524	Betherur	Kod
			1526	Mandihal	Dharwar
			1528	Pale	Hubli
			1530	Kodur	Kod
			1533	Wanhalli	Dharwar
			1534	Hullur	Kod
			1536	Bomanhalli	Hangal
			1537	Surleswar	"
			1538	Mulgund	"
			1539	Lakhamapur	Karjgi
			1540	Nittur	Ranebenur
			1541	Konantalli	"
			1544	Mangoor	Chikodi
			1546	Belanki	Athani
			1550	Devguri	Karjgi
			1551	Domihal	Kod
			1553	Malnaikanhalli	Ranebenur
			1554	Ugar	Athani
			1557	Aurwar	Chikodi

APPENDIX D- *concl'd*

Registered number	Place	Taluka	Registered number	Place	Taluka
<i>October 1911 to 1919</i>			<i>July 1917 to 1919 3rd Series—concl'd</i>		
1603	Shiswinhalli	Bankapur	1758	Masangi	Kod
1607	Muralli	Hubli	1761	Malhalli	Hubli
1608	Marimagalpur	Sampgaon	1766	Kandagi	Hangal
1610	Karguppi	Hukeri	1767	Kodni	Chikodi
1612	Yerguppi	Hubli	1768	Ganjigatti	Kalghatgi
1615	Hawa-shi	Karjgi			
1616	Yellapur				
1617	Itagi	Khanapur			
1618	Adi				
1619	Sampgaon	Sampgaon	1801	Ganjigatti	Kalghatgi
1620	Dombarkop		1804	Adi	Khanapur
1623	Mulgund	Hangal	1805	Nichanki	Sampgaon
1624	Karemandalgi	Ranebenur	1806	Thamhatarg	Hukeri
1628	Chikkerur	Kod	1807	Gaurwad	"
1629	Hawashibhavi		1809	Domihal	Kod
1631	Totad-elapur	Karjgi	1810	Betherur	"
1632	Yelliwal	Hangal	1813	Garlegunji	Belgaum
1633	Hulatti	Kod	1814	Kerwad	Khanapur
1634	Ramankop	Bankapur	1815	Bidi	"
1635	Keshavapur	Hubli	1816	Halgi	"
1636	Yedur	Chikodi	1817	Anantpur	Athan
1637	Benadi	"	1818	Nichanki	Sampgaon
1638	Chandur	"	1819	Mallapur	Gokak
1639	Talawagi	Khanapur	1820	Masalmari	"
1640	Aigali	Atham	1821	Gondikop	Chikodi
1641	Mulgundi	"	1822	Hiremmagdur	Karjgi
1642	Nesargi	Sampgaon	1823	Belgalpetli	Hangal
1646	Halgeri	Ranebenur	1826	Kyalkop	Dharwar
1647	Chikmagnur	"	1827	Kemur	Nargundpetha
1648	Murali	Bankapur	1829	Chiknargund	"
1649	Parwatgeri	Kod	1830	Bisalhalli	Ranebenur
1652	Yedur	Chikodi	1834	Kod	Kod
1655	Thamha targi	Hukeri	1835	Somanhalli	"
			1836	Hoslati	"
			1837	Sididevarkop	Karjgi
			1838	Kabbur	"
			1839	Hamsagar	Hangal
			1840	Benankatti	Dharwar
			1841	Ramankop	Bankapur
			1845	Mangali	Athru
			1848	Hidhal	"
			1849	Naudi	Chikodi
<i>July 1917 to 1919, 3rd Series</i>					
1715	Domharkop	Sampgaon			
1720	Gudjernal	Gokak			
1725	Kakur	Mundargipetha			
1730	Kopgondankop	Hangal			
1731	Hanamsagar	"			
1755	Mudengudd	Ron			

THE SPOROZOITE RATE OF ANOPHELINES CAUGHT WILD IN THE TERAI 1931

BY

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AND

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WITH

A NOTE ON SOME CORRELATIONS IN THE INFECTIVITY OF
*A FUNESTUS**

BY

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DURING the League of Nations' Malaria Commission's tour of India, the value of what we have called the Sergeants' method for determining the malarial infectivity of mosquitoes was suggested to us by Professor Dr Schuffner, and shortly afterwards we tested its efficacy in the laboratory, as has been described in a paper on the subject by Strickland and Roy (1931†)

The assessment of the influence of any factor in an endemic disease should of course be based on such a number of observations as are, as far as practically

* This title has been retained for the Anophelines otherwise known as *minimus* and *histoni*

† We were not then aware that the method had been referred to in a paper by Soesilo (1928)

possible, free from the error of random sampling, which being so, a reliable evaluation of the infectivity-rate of an Anopheline species would, if one follow the tedious and old-fashioned methods of isolating the salivary glands and gut of the mosquito, involve considerable expenditure of time and therefore of money, and we saw in the new method an inexpensive way of ascertaining the value of this factor, perhaps the most important in malarial endemiology

We therefore conducted the inquiry here reported upon—

(a) to see how the method could be utilized as it might be in a routine public health survey, and what would be the value of such results as we would obtain having regard to the probable errors inherent in the data collected,

(b) to ascertain the local variations in the Anopheline infectivity-rate, outside of any limits of probable error, and to calculate the degree of correlation between these variations and some of the coincident physical conditions, and

(c) to study the habits of the adult Anophelines

ORGANIZATION AND METHODS

We chose the Darjeeling Terai (*see Map*), one of the most highly endemic malarial areas of India, the tea-gardens in this tract having a spleen-index of 75 per cent, as the scene of our inquiry

One of us (K. L. C.) took charge of the field-laboratory at Siliguri, and Mr. McGregor under him was, for most of the time, in charge of the collections in the field. Unfortunately the former became seriously ill with malaria for a month, when Dr. D. N. Roy, Assistant Professor of Entomology with Colonel Acton's kind permission, took his place

Our first plan was to visit the same parts of the Terai in rotation once every month, so that we should be able to observe any monthly variation in the infectivity-rate in the same set of localities. For various reasons however, chiefly on account of lack of our own motor-car transport, our programme could not be strictly adhered to. Then to add to our own collections we tried to obtain a regular supply of adult mosquitoes from the local tea-gardens, and we issued a circular describing how to collect and to send the mosquitoes alive in Barraud's boxes to our laboratory at Siliguri. We also wished to study the coincident gametocyte rates in the different parts of the Terai, and issued another circular to the tea-gardens describing the technique of taking thick and thin blood films. To this request the response was unfortunately poor, so that we cannot show in this report how the gametocyte rates were related to the infectivity-rates that we obtained. Our own staff was not sufficient to undertake this side of the inquiry.

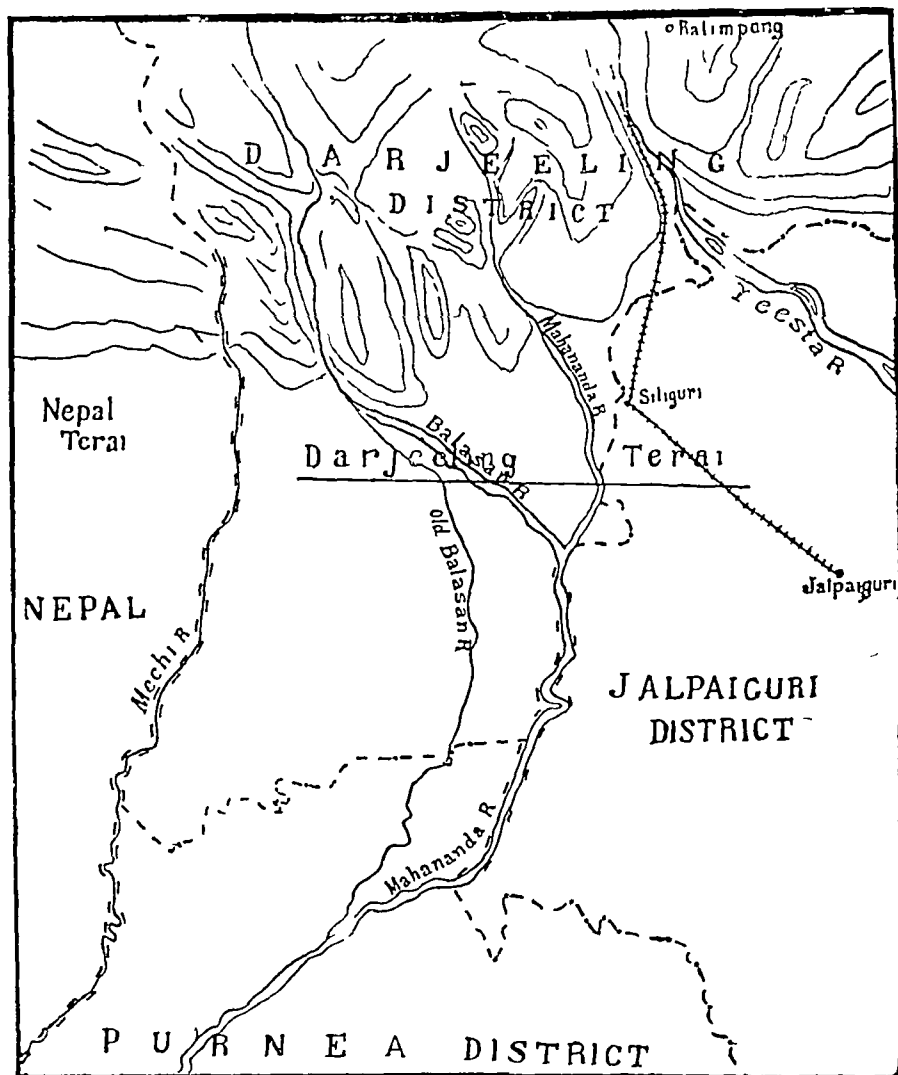
The collection of the adult Anophelines was made in the morning, from about 6 to 10 a.m. and in the evening from about 4 to 9 p.m.

In tea-gardens there was not much difficulty in obtaining entrance into coolie-huts, as the collectors were always accompanied by a garden *choulidar**. We had

* Watchman

more trouble in getting into houses in *bustees*^{*}, despite the fact that the bustee choudkars over an area of 14 square miles had been warned of our coming by the Deputy Commissioner. The bustee coolies, where admission was refused, spoke of

MAP



purdah], but the real reason would seem to be that they suspected we belonged to the Excise Department, investigating the illicit manufacture of liquor

* Villages

† The seclusion in which certain classes of Indian women are kept

Generally speaking, however, after a little persuasion we gained admittance to most houses¹

Two hundred female *Anophelines* were daily required for dissection. But the daily collection varied a good deal according to the locality visited and the weather, and our catch was prejudiced because this year during the rains there happened to be an excessive and adverse rainfall. Not counting a period when there was a complete cessation of work the observations extended over 20 weeks, and the average catch per diem was about 130 as against the 200 originally estimated for. This shortage was due not only to the prejudicial rains but also to certain initial difficulties in organization and transport arrangements and later to illness which upset the organization considerably.

There really turned out to be no potential reason why, even with the reduced staff that we employed we could not have easily obtained and examined the 200 female *Anophelines* per diem that we had estimated for. Dr Roy examined 2,165 in one week, viz., 361 per diem, and in the best days work he examined 423.

The morning collection was examined in the afternoon, and the evening collection the next morning. The method of examination was first to group the catch into different species, then laboratory assistants would make the preparation of the 'body-slides' from 5 of the mosquitoes at a time, placing the cover-slips in a line so that they could be run over quickly along one axis of the moving stage. The result was recorded on cards, on which was also shown the place of collection according to the following schedule —

- (1) tea-garden coohe-huts,
- (2) tea-garden bungalows (superior staff)
- (3) tea-garden 'bashas' (clerks' quarters)

¹ Notes by Dr McGregor on methods of collection of mosquitoes —

¹ The plan followed, though not always adhered to, was that the bustees were visited in the mornings, and tea gardens in the evenings, because in the morning tea garden labourers were out early into the gardens and closed their huts behind them.

¹ The bustees visited were within a radius of about ten miles of Siliguri, tea gardens, in one case, as far as twenty miles. Bustees were taken at random, tea gardens, as far as possible, in the order in which they were visited during the first month of the work.

¹ One "Petiomax" lamp was in use, morning and evening. On arrival at the place to be searched, the whole collecting staff of five, the saidar (foreman) and 4 men, would go inside each hut in turn. Occasionally, the party would divide, if any hut nearby could be worked without a lamp. On entering a hut, the first thing done was to hang the lamp in the centre of the hut, then care being taken not to disturb any hanging clothes, empty receptacles, etc., the catchers would go to the four corners, and commence to examine these, working along the walls. Where possible one man would mount one of the cross bars, and examine the roof. Then the walls and roof having been examined, hanging clothes, empty barrels, pots, etc., would be searched.

¹ So long as mosquitoes could be found in good numbers, the search continued, until there was a falling off. Then a move would be made to the next hut. Perhaps here, mosquitoes would be few, and the number caught out of all proportion inversely to the time given. If so no more time would be wasted, and a move made to another hut. Often a bustee or a tea garden coohe line would produce so few mosquitoes, that we would return to our motor car and move to another point. One of the catchers being of a low caste, was often refused admittance to huts, when he would be set to examine the cow-sheds, always close by. Following the above procedure, the party would return when all the test tubes taken out had been used up.

¹ Mosquitoes were caught, one or two to each test tube. On each tube was written with a glass pencil, a number, signifying the type of place in which it had been used. On our return the tubes of each number were sorted out into boxes, and were then dealt with by the laboratory assistants.

- (4) bustees (villages),
 (5) cattle-sheds in tea-garden coolie-lines,
 (6) cattle-sheds in bustees

SUMMARY OF OUR DATA

The number of Anophelines collected over the whole period from the 20th April to 26th September, 1931, was 25,904

Of these, 22,049 were females and 3,855 males of the species shown in Table I, which also gives the result of the examination of the females for the sporozoites —

TABLE I

Anopheline species	Total caught	Examined by the Sergents method Females *	Number showing sporozoites	Probable error	Gross rate per cent of infection	Probable error in gross percentage rate
<i>aconitus</i>	8	8	Nil		Nil	
<i>barbirostris</i>	3	3	"		"	
<i>culicifacies</i>	1,707	1,138	"		"	
<i>fuliginosus</i>	1,556	1,417	"		"	
<i>funestus</i>	8,302	7,912	308	± 11.4665	3.89	± 0.14
<i>hyrcanus</i> var <i>nigerrimus</i>	15	15	Nil		Nil	
<i>jamesi</i>	21	19	"		"	
<i>jeyporiensis</i>	7	7	"		"	
<i>loch</i>	1	1	"		"	
<i>maculatus</i>	71	55	"		"	
<i>maculipalpis</i> var <i>indiensis</i>	332	289	"		"	
<i>philippinensis</i>	30	29	1	± 0.6627*	3.4	± 2.28‡
<i>subpictus</i>	} 13,851	704	Nil		Nil	
<i>vagus</i> †		10,452	2	± 0.942	0.019	± 0.009§

* The unexamined specimens of the total catch were males

† This probable error in the gross per cent infection rate, indicates that in 50 per cent of similar trials, the infection rate would be found to be between 3.75 and 4.04 per cent. In 96 per cent of similar trials the resultant rate would fluctuate between 3.47 and 4.31 per cent, a rather considerable divergence for, from a practical point of view, such a large sample.

‡ The sample being small this probable error is not to be regarded as reliable.

§ Though this probable error is relatively to the gross infection rate great (compare *funestus* above), it is still significant, and therefore *vagus* must be reckoned to have definite potentialities as a vector.

ERRORS IN VARIOUS DEGREES OF SAMPLING DURING OUR SURVEY

As *A. funestus* was the only species^r in the above list that showed any considerable infectivity-rate, we have analysed the figures for this species only

For the analysis we have taken the data for —

- (A) the whole period,
- (B) the alternate days of the survey,
- (C) the alternate days of alternate weeks

(A) *Probable errors in the samples taken for the whole period*

7 912 *funestus* were examined, of which 308 were found infected

The probable error of this number of infections was $\pm 11\ 4665$

The gross rate per cent of infection was 3 893

The probable error per cent was $\pm 0\ 1467$

so that in 50 per cent of trials the infection-rate would be found to lie between the limits of 3 75 per cent and 4 04 per cent

(B) *Probable errors in the samples taken over the whole period on alternate days of the survey*

Series	Number dissected	Number infected	Gross infection rate, per cent	Probable error, per cent	Probable limits of deviation from gross per cent infection rate
1	4,359	171	3 923	$\pm 0\ 1972$	3 73 and 4 12
2	3,553	157	3 856	$\pm 0\ 2156$	3 64 and 4 07

(C) *Probable errors in the samples taken over the whole period on alternate days of alternate weeks*

Week series	Day series	Examined	Sporozoites in	Probable lower limit of deviation, per cent	Gross infection per cent rate	Probable higher limit of deviation, per cent
1	a	1,922	81	3 915	4 215	4 515
	b	1,775	73	3 84	4 11	3 38
2	a	2,437	90	3 44	3 70	3 96
	b	1,778	64	3 22	3 60	3 98

* *philippinensis*, which also showed a high gross infective rate, must obviously be excluded from consideration because of the few specimens examined

This analysis of the probable errors in the *funestus* infection-rates yielded by the samples we took shows that from a practical point of view (a) when these numbered about 2,000 they had probable errors which were comparatively great, and (b) a sample of about 8,000 yielded a probable error not very much less.

If all of our mosquitoes viz 22,019 had been of one species and had given us the same gross infection rate as *funestus* viz 3.89 per cent the probable error in this rate would have been ± 0.088 per cent while the same number of mosquitoes giving gross infection-rates of 50 per cent and 99 per cent would have had probable errors of ± 0.0227 per cent and ± 0.0452 per cent respectively.

VARIATION IN THE INFECTIVITY RATE OF *A. funestus* IN THE TERAI

We show below the infectivity-rates of *funestus* at different periods of the survey —

(a) in two main divisions of the total period, and

(b) in four periods

(a) The following were the results of this analysis —

	20th April to 27th June	17th July to 26th Sept
Specimens examined	3,486	4,426
Specimens infected	123	185
Gross infectivity rate per cent	3.5	4.1
Probable error per cent	± 0.21	± 0.20
Mean daily rainfall	0.61 in	0.75 in
Mean daily maximum temp	86.04°F	88.80°F
Mean daily minimum temp	74.00°F	75.25°F
Mean of weekly means of relative humidity*	87.05	95.41

In the latter half-period one *philippinensis* out of 17, giving a rate of 5.8 per cent, and 2 *vagus* out of 7,122, giving a rate of 0.028 per cent, were also found infected.

(b) The following were the rates in the four periods —

	20th April to 16th May	17th May to 27th June	17th July to 15th August	16th August to 26th Sept
Number examined	464	3,022	2,634	1,792
Number positive	17	106	125	60
Gross infectivity rate per cent	3.66	3.2	4.7	3.3
Rainfall, inches	3.38	35.93	43.73	23.79
Maximum temp (mean)	84.39°F	87.69°F	88.07°F	89.54°F
Minimum temp (mean)	75.86°F	72.13°F	74.54°F	79.95°F
Relative humidity (mean)*	85.70	88.40	95.73	95.10

* The humidity figures were those for Kalimpong and were supplied by the Alipore Observatory. That station is not in the Terai but in the adjoining foothills (see Map).

It was in the third period that *vagus* and *philippinensis* also were found infected

The last table exhibits a very striking rise of the infectivity-rate during the third period, which roughly corresponds with the establishment of the rains* Dr Chaudhuri's note on the subject (*vide* Appendix) shows that humidity, minimum temperature, and rainfall, had each, within the limits of the observations some positive influence. Their combined effect was very significant; the maximum temperature played an inverse part, but as the inverse influence of this factor in the case of the infectivity-rate of the third period, was only equivalent to about 0.1 per cent infectivity, the considerable rise in the rate in this period must be ascribed to the combined favourable influence of the other factors.

VARIATIONS IN INFECTIVITY IN DIFFERENT TYPES OF HABITATION

The following were the results of the analysis of *funestus* infectivity according to the type of habitation from which the mosquitoes were collected —

	Examined	Infected	Rate	Probable error, per cent
Tea garden coolie houses	4,557	204	4.7	± 0.206
Bashas	16	0		
Bungalows	2	0		
Tea garden coolie line cattle sheds	4	0		
Bustees (villages)	3,136	99	3.0	± 0.210
Bustee cattle sheds	198	5	2.5	± 0.75

There was therefore a true and great superiority of infectivity of *funestus* caught in tea-garden coolie-habitations as compared with those caught in bustee habitations.

This has a very important bearing on the controversy whether it is better to have garden-coolies living in 'lines' on an estate, or to let them live in bustees outside (*faltoo* coolies). No reason for the difference shown can be, at the moment, suggested, and it would be an important matter to attempt to discover the possible factors involved.

* This was also Dr Bentley's finding in Bombay in relation to *A. stephensi*.

HABITS OF THE ANOPHELINES

In another paper Strickland and Chowdhury (1931), dealing with the epidemiology of blackwater fever in the Terai, suggested that a study of the habits of adult Anophelines particularly those of *funestus* and *maculatus*, might yield some explanation of the low incidence of that disease in tea-garden coolies as compared with those of higher social grade*, and the following observations have some bearing on this subject.—

The relative number of adult Anophelines caught morning and evening

Table II shows the relative numbers, the time spent in the mornings being on the average a little longer than in the evenings —

TABLE II

	Morning catch (bustees)	Evening catch (tea gardens)
<i>funestus</i> (minimum and maximum)	746	1,103
<i>vagus</i>	2,068	850
<i>rossi</i>	14	29
<i>culicifacies</i>	3	11
<i>philippinensis</i>	4	3
<i>maculipalpis</i>	2	1
<i>maculatus</i>	1	3
<i>fuliginosus</i>	45	2
TOTALS	2,883	2,002

However, as the morning and evening catches were made, *as a rule*, in different types of habitation, viz, in bustees and in tea-gardens respectively, we will not attempt to interpret the figures

Sheltering places of the adult Anophelines and their zoophilism

The number of adults of each species that the various types of habitation harboured is shown in Table III, but these types should not be compared *inter se*

* Malarial 'immunity' appears to have no influence in the matter

as the different dwellings were not searched for equal periods of time, nor at the same time of day or night

TABLE III

	Tea garden coolie lines	Bashas	Bungalows	Bustees	Tea garden cattle sheds	Bustee cattle sheds
<i>barbirostris</i>				1		2
<i>culicifacies</i>	383	1	1	382	12	359
<i>fuliginosus</i>	95	2	1	153	22	1,144
<i>funestus</i> and <i>aconitus</i>	4,558	16	2	3,138	4	198
<i>hyrcanus</i> var <i>neglensis</i>						15
<i>jamesi</i>	5					14
<i>jeyporiensis</i>				3		4
<i>loch</i>						1
<i>maculatus</i>	30			5	1	16
<i>maculipalpis</i>	96			65	9	119
<i>philippinensis</i>	11			9	1	8
<i>subpictus</i>	288	4		258	21	133
<i>vagus</i>	3,891	27	23	5,788	17	706

One can, however, fairly utilize the table to compare one species with another in so far as their ratio in different types of habitation varied. Thus, the following was the approximate relative prevalence of the more important species in human habitations and cow-sheds —

TABLE IV

	Human habitations, per cent	Cow sheds, per cent
<i>funestus</i> and <i>aconitus</i>	98	2
<i>vagus</i>	93	7
<i>rossi</i>	78	22
<i>culicifacies</i>	68	32
<i>maculatus</i>	64	36
<i>fuliginosus</i>	18	82

In this schedule the species have been arranged in order of their relative zoophilism and the remarkable relative zoophilism of *fuliginosus* stands out well, as also the corresponding homophilism of *funestus* and *vagus*, while *maculatus* and *culicifacies* occupy a relatively intermediate position.

Our experience was that, if cattle-sheds had walls with only a small door as an entrance the inside was dark and malodorous, and many mosquitoes, principally *culicifacies* and *fuliginosus* could be found. If, however, it had only a roof on supports and no walls, a few or no mosquitoes were found, while the adjoining coolie-huts would harbour many. Most mosquitoes were found in human habitations (whether in coolie-lines or in bustees) when there were animals near, as for instance goats in pens.

Mr McGregor was of the opinion that in spite of the unequal opportunity of catching afforded in the several types of habitat, mosquitoes were not really so numerous in the houses of tea-garden managers and clerks as in those of coolies, and as his main object was to collect mosquitoes for dissection, he went where they could most readily be found, and hence that opinion was probably correct. His conclusion of course should not be wondered at, for generally speaking on the one hand the habitation of the manager and clerk is light, clean, airy, and pucca, on the other the coolie's hut is dark, air-stagnant, untidy, and kutchha*. In spite of its smokiness it harbours many mosquitoes.

Parts of habitations affected by the Anophelines

Whatever be the relative attraction in the Terai of the different types of habitation, there was no particular part of any dwelling where the Anophelines were more commonly found: they were captured equally from walls both high up and low down, and many were found resting on spider-webs and on straws hanging from the roof. In one hut a large pile of wood in a dark corner harboured many *funestus*.

In managers' and clerks' houses Anophelines were often found on or inside mosquito-nets and on their supports. The nets usually seemed to be carelessly rolled up if at all, while in Chinese mistris'† quarters they were usually hung up open and the mosquitoes found inside the nets.

Hanging clothes seemed to be neither particularly attractive nor repellant to Anophelines. In one bustee hut in which clothes were hanging 56 mosquitoes were taken, and of these about 15 were of the *rossi-vagus* group and 41 *funestus*, but the mosquitoes were not resting particularly on the clothes. In an adjoining hut, exactly

* At Noamundi on the other hand we found (Strickland and Chowdhury, 1930) that clerks' quarters were far more productive of Anophelines than coolies' but the relative character of the habitations differed from that pertaining in the Terai. At Noamundi while both the coolie quarters and the clerks' quarters were pucca, light and airy, the coolie lines were always full of smoke, the clerks' quarters were seldom smoky. The smoke probably prevented Anophelines appearing in the former, while there was seldom smoke to prevent them entering the latter.

Why should there have been few Anophelines in the clerks' quarters in the Terai and many at Noamundi? In both cases the habitations were light and airy. It would seem to be difficult to explain this. Possibly it was some greater attraction of the kutchha coolie buildings. On this assumption the relative importance of the main characteristics of the habitations concerned in order of their greatest attraction would be —

'kutchha'ness, 'pucca'ness, smokiness

† Artisans'

similar in all respects except that in it no clothes were hanging, 17 mosquitoes were caught, all of the *rossi-vagus* group. Perhaps *funestus* appreciates the human effluvium. Bath (1931) says that Anophelines do

Shelters of Anophelines outside human habitations

Do the Anophelines in the Terai after feeding on man shelter inside his habitations or cow-sheds, to digest their food (incidentally developing their parasites), pending their visits outside to lay their eggs, or do they shelter outside?

We have shown above that more infected mosquitoes were found in the evening (tea-gardens) than in the morning (bustees). This difference may possibly have been due to the fact that the older and therefore more highly infected mosquitoes had left the human habitations for oviposition at about dawn before the usual time for our morning collection and returned for food before our evening collection. That would have accounted for a higher infective-rate in the evening than in the morning because there would be a larger proportion of young and uninfected mosquitoes left behind in the morning.

It has been observed that in Africa *funestus* has the habit of sheltering among great boulders of river-beds, but in the Terai we could not get any confirmation of this observation. We searched along the bed of the Balasun river for resting-places of adult Anophelines which as larvæ are found in great numbers there but they were neither caught in the vegetation nor among the rocks strewn along the bed.

RELATIVE PREVALENCE OF THE SPECIES AND SEXES

The list of species taken as adults over the whole period of our survey is given in Table I (q v)

The species at different seasons

During the half-periods of our survey the following was the relative prevalence of the different species:—

	April to June	July to Sept
<i>aconitus</i>	7	1
<i>barbirostris</i>	1	2
<i>culicifacies</i>	1 681	26
<i>fuliginosus</i>	1,274	282
<i>funestus</i>	3 729	4,573
<i>hyrcanus</i> var <i>nigerimus</i>	14	1
<i>jamesi</i>	18	3
<i>jeyporiensis</i>	6	1
<i>koehi</i>	1	0
<i>maculatus</i>	62	9
<i>maculipalpis</i> var <i>indicus</i>	317	15
<i>philippinensis</i>	12	18
<i>subpictus</i>	} 5,232	8,619
<i>vagus</i>		

Therefore *culicifacies fuliginosus*, *maculatus* and practically all the minor species were found in greater numbers in the drier period, the decrease of *culicifacies* during 'the rains' being most remarkable. On the other hand *funestus* and the *vagus-rossi* group were much increased.

Relative prevalence of the series

The sex ratios over the whole period are shown in Table I, of all the species, 22,049 females and 3,855 males were caught. Of *culicifacies* about one-third were males, of *fuliginosus* about 9 per cent, of *funestus* about 5 per cent, of *maculatus* about 24 per cent, and of the *rossi-vagus* group about 20 per cent.

If a greater proportion of males to females of a species in any place is an indication that its breeding place is correspondingly nearer, then *culicifacies*, *maculatus* and *vagus* were breeding closer to the habitations than *fuliginosus* and *funestus*. This however was certainly not the case, so that the hypothesis derives no support from our survey.

Relative sex rate at different seasons

When those species that were caught in any considerable numbers during the two periods, viz. the dry weather and the rains, were compared, it was found that the percentage of males in the total catch was as follows —

TABLE V

	In the dry weather period, per cent	In the rainy period per cent
<i>fuliginosus</i>	10	3
<i>funestus</i>	6	3
<i>vagus</i>	25	17

This table shows a lower average rate of males caught during the rainy season, in both 'rains' breeders and dry-weather breeders.

Relative sex rates per type of habitation

A higher proportion of male specimens of *culicifacies* and *funestus* was found in 'bustees' than in the tea-garden coolie-houses, whereas in the *vagus* group the reverse was the case.

The influence of fires and smoke in habitations on the prevalence of Anophelines

Some observations were made on this side of the subject chiefly in so far as coolie-huts were concerned.

In this connection we reiterate that at Noamundi the coolie-lines as well as clerks' quarters were built of stone, but whereas in the former they were always

full of smoke and the walls smoke-blackened in the latter the rooms were free of smoke. It was in the latter that by far the greatest proportion of the mosquitoes were caught and it was among the occupants of the latter that blackwater fever was prevalent.

We will now compare these conditions with those we found in the Terai. Here the coolie-huts were generally speaking clean, those in the 'bustees' particularly so. They nearly all had a fire-place built inside for cooking, but in spite of this the interior appeared to be less smoked than the *pucca* buildings at Noamundi, probably the ventilation through the thatched roof or under the eaves, was good. We noted how free from smoke many huts were although cooking was habitually carried out in them. In any case coolie-huts generally speaking harboured a large number of Anophelines, and it is fair to presume that the degree of smoke in them was not sufficient to prevent mosquitoes entering in such numbers as appeared to be the case at Noamundi. When there was considerable smoke in a hut mosquitoes were rarely found, and when there was no smoke more mosquitoes were found. Fires in the Terai tea-gardens were usually lighted at dawn for making tea, but in the bustees fires were lit later in the morning while in both they were lighted at dusk for the evening meal.

We believe, as stated elsewhere (Strickland and Chowdhury, 1931), that smoke is a very important factor in the control of Anophelines in human habitations. The smoke from the fire probably kills off a very large number of the Anophelines that have entered the houses.

The species *vagus* was usually found in huts in which no fires had been lit for some time, so that it is possible that it is more sensitive to fumigation than *funestus*. *funestus* on the other hand was oftener found in huts when the fires were being used, and burning brightly, it appeared to be attracted by warmth. Whenever found in large numbers (perhaps accompanied by a few *vagus*) it was nearly always in a house with a bright clear fire giving off no smoke.

Reaction to the illumination of habitations

European and clerks' habitations generally speaking were well lighted, as compared with coolie-huts, and in the former Anophelines were relatively very scarce. Certainly coolie-huts, without any light at all or with only a glimmer coming in between the eaves of the thatch-roof and walls, would often harbour hundreds of mosquitoes.

In the case of certain species the *rossi-vagus* group appeared to affect the better lighted huts rather than the darker, whereas the reverse was the case with *funestus*, *maculipalpis* and *maculatus*. In one hut a great number of *funestus* were found massed in a corner where it was darkest. If there be any such difference, as indicated, the greater catch of *vagus* in the morning could be accounted for on this ground only.

SUMMARY

The inquiry was undertaken to study the applicability of the Seigents' method of detecting sporozoites in the mosquito to the requirements of Public Health Services.

(2) About 25,000 mosquitoes were examined and *A. funestus* ('*minimus*' chiefly) was found to be mainly responsible for the very high malaria-rate in the Terai. *A. vagus* also had a significant infection-rate.

(3) The gross sporozoite rate of *funestus* was about 4 per cent. However, on analysing the probable error not only in this figure but also in the infectivity-rate of samples of decreasing size that were taken during our inquiry, we found that from a practical point of view the probable errors were considerable.

(4) The variation in the gross infection-rate of *funestus* that we found at different periods was apparently related with the onset of the rains, but otherwise not with the main meteorological factors prevailing. Dr H P Chaudhuri, however, on analysing our data, found a significant relationship with the minimum temperature, as well as with humidity, and rainfall, the maximum temperature on the other hand had an adverse influence.

(5) We also found a significant difference between the infection-rates of *funestus* caught in tea-garden huts, and of those caught in the villages.

(6) The habits of the local species have been discussed. The relative homophilism of *funestus* was very marked.

CONCLUSION

The relative danger in the Terai of the different species is expressed in the relative numbers of those found infected, thus —

	Number of the species found infected with sporozoites
<i>funestus</i>	308 probable error ± 11.47
<i>vagus</i>	2 „ „ ± 0.94
<i>philippinensis</i>	1 „ „ ± 0.67

The relative danger of the one species, *funestus*, in the houses of coolies in tea-gardens, and of villagers in their bustees, is shown in the following table, on the assumption that the relative numbers of this species that we obtained in these two types of habitation represented the truth, and were not biased by the fact that the catches were respectively made in the evening and morning.

funestus infection

	Numbers examined	Rate per cent	Relative danger of <i>funestus</i> about
Tea gardens	4,557	4.7	214
Bustees	3,136	3.0	94

As stated above, if the latter table represents the truth it would account for much of the coolie's insistent wish to leave tea-garden lines, and live in the bustees

Above we have asked the reader to accept as correct the conclusion that in the Terai there are more mosquitoes in coolie-habitations than in the houses of those of higher social position. From this it would follow that the malaria inoculation rate in the former is proportionately higher. Yet we have seen (Strickland and Chowdhury, 1931) that the blackwater fever incidence is very much lower among the coolies, than among Europeans, Bengalis and Chinese. What is the controlling factor? Smoke, cows? We suggested both possibilities. As for cows, we have shown above that they divert from man the attention of *funestus* less than any other species, so much so that this species is comparatively rare in cow-sheds. We therefore think that this factor is unimportant, when compared with the results of the fumigation from the coolie's fire. If cattle do not attract *funestus* to any great extent we believe that the coolie although living in a 'funestus house,' does not get blackwater fever because of a great destruction by smoke of the infected mosquitoes whereas in non-fumigated houses there is no such destruction.

It is an interesting commentary on the phrase 'blackwater house' that Mr. McGregor, our field-assistant, became accustomed to refer to certain coolie habitations as 'funestus houses', i.e., meaning that he could always collect a good number of this species in these houses. As we have pointed out previously (1931) a blackwater house in the Terai is probably a non-fumigated house, and also a *funestus house*.

ACKNOWLEDGMENTS

We wish to thank the Indian Research Fund Association for kindly putting the necessary funds at our disposal, Colonel H. W. Acton, C I E, I M S, Director, School of Tropical Medicine and Hygiene, Calcutta, for permitting Dr. Roy to fill the breach caused by the illness above referred to, Colonel (now Sir) Hassan Suhrawardy, M D, C M O, Eastern Bengal Railway, for his help in every way possible, and for the co-operation of his staff at Siliguri, especially Drs. P. Sinha and I. Ahmed, and finally, Mr. H. W. Cox, the Chairman, and other members of the Terai Planters' Association for their assistance and kind hospitality. Also in the preparation of this report we are very much obliged to Dr. H. P. Chaudhuri for kindly helping us with working out the probable errors in the gross rates calculated from our material.

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APPENDICES

- I THE PRACTICABILITY OF SUCH A SURVEY AS A PUBLIC HEALTH SERVICE PROCEDURE
- II A NOTE ON SOME CORRELATIONS IN THE INFECTION RATE OF *A. funestus* BY
H P CHAUDHURI, M B (Cal), D P H, D T M & H, F R S S (Lond)

APPENDIX I.

THE PRACTICABILITY OF SUCH A SURVEY AS A PUBLIC HEALTH SERVICE PROCEDURE

We can say at once that we have demonstrated the practicability of such a survey of ours as part of a public health service. Allowing for observations to be carried on for a year so as to study differential infectivity at seasons, we would create an organization to be placed in charge (part-time) of existing Health Officers. The additional staff required would then be, it is suggested —

	Rs per month
I <i>The catchers</i> —	
one sardar @	40-0-0
four men @ 15/-	60-0-0
II <i>The laboratory staff</i> —	
two assistants who could be taught to identify the mosquitoes and the sporozoites @ 40/-	80-0-0
two menials for preparing the slides by the Ser-gents' method @ 20/-	40-0-0
Transport	60-0-0
Contingencies	20-0-0
	<hr/>
TOTAL	300-0-0
Per annum Total Rs	3,600-0-0

This organization would suffice to work out the infectivity of the Anophelines of a district based on at least 60,000 specimens per annum

APPENDIX II

A NOTE ON SOME CORRELATIONS IN THE INFECTION-RATE OF *A FUNESTUS*

BY

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With a view to seeing whether there were any relationship between the malaria infection-rate of *Anopheles funestus* in the Darjeeling Terai and certain of the local physical conditions, viz, rainfall, humidity, maximum temperature, and minimum temperature, I have utilized the data placed at my disposal by Drs Stuckland and K L Chowdhury as collected by them in the course of the survey, reported on herewith, and the results of my analysis are given below. I am much obliged to them for the opportunity of making

this review and I must also express my gratitude to Lieut-Colonel A D Stewart, I M S, for his guidance and encouragement in analysing the matter, and for his advice in submitting this report

Material and methods

With extensive material there is but little danger of false conclusions being drawn, whereas with such comparatively few cases as those at my disposal, an uncritical application of the correlative method might be so misleading as to endanger the credit of this most valuable weapon of research

It is not true, however, that valid conclusions cannot be drawn from small samples. If particular care be taken to pay due regard to the 'probability' of the data leading to any conclusions to which we may come, our judgment will be a fair one

In my present analysis, therefore, I have tried as far as possible to keep myself within the boundary-walls of the accurate scientific tests, and in my conclusions the amount of significance to be attached to each is reflected in the values of their 'probability', which I have in each case shown

The period of observations, from 20th April to 28th June and from 17th July to 25th September, 1931

The place of observation, the Darjeeling Terai

Prevailing physical conditions

Maximum temperature	84.5°F to 95.5°F, average 87.42°F
Minimum temperature	69.00°F to 81°F, average 74.62°F
Rainfall per week	0.83 to 19 inches, average 4.8 inches
Relative humidity*	73 to 89 per cent, average 83.30 per cent

Methods

I have tried to show the relationship of the infection-rates with these meteorological factors, through —

- (i) the correlation coefficients,
- (ii) the regression coefficients,
- (iii) partial correlations, and by
- (iv) diagram

(1) *Correlation coefficients (r)*

No quantity is more characteristic of modern statistical work than the correlation coefficient, and no method has been applied more successfully to analysis of very

* At Kalimpong in the foothills adjoining the Terai (see Map)

various data than the method of correlation. Observational data have been given by its means an altogether new importance.

The following are the correlation coefficients (r) obtained from the data —

(1)	r of percentage of infection with maximum temp	— 0.1152 ± 0.14877
(2)	„ „ „ „ minimum temp	+ 0.4002 ± 0.12662
(3)	„ „ „ „ rainfall	+ 0.29505 ± 0.137675
(4)	„ „ „ „ humidity	+ 0.3132 ± 0.1357
(5)	„ „ „ „ „ mean temp	+ 0.214 ± 0.144

From these coefficients it will be seen that —

(1) The relationship between the percentage of infection and the maximum temperature is insignificant and inverse viz, — 0.1152 ± 0.14877

The probability that this coefficient may arise from random sampling in an uncorrelated population is more than 60 per cent

(2) The relationship between the percentage of infection and the minimum temperature is significant, being + 0.4002 ± 0.12662 and the probability that it may arise by random sampling from an uncorrelated population is only 7 per cent

(3) The relationship between the percentage of infection and the amount of rainfall is not insignificant it is + 0.2951 ± 0.137675 and the probability that it may arise by random sampling from an uncorrelated population is 20 per cent

(4) The relationship between the percentage of infection and the amount of humidity is not insignificant and is + 0.3123 ± 0.1357 and the probability that it may arise by random sampling from an uncorrelated population is 17 per cent

(ii) *Regression coefficients (r')*

The idea of regression is usually associated with the theory of correlation, but it is in reality a more general and a simpler idea, and the regression coefficients are of interest and of scientific importance in many classes of data where the correlation coefficient is an artificial concept of no real utility.

In the present case the following are the regression coefficients (r') that I have obtained —

(1)	r' of infection with maximum temperature	— 0.0710
(2)	„ „ „ „ minimum temperature	+ 0.2134
(3)	„ „ „ „ rainfall	+ 0.1107
(4)	„ „ „ „ humidity	+ 0.1241

From this schedule it will be seen that the regression coefficients of —

(1) the percentage of infection to the maximum temperature is 0.07, i.e., for a rise or fall of every degree of maximum temperature away from 89.95°F and

between 81.5°F and 95.5°F , there will be 0.07 per cent decrease or increase of infection

(2) the percentage of infection to the minimum temperature is $+0.2134$ per cent increase or decrease of infection for every degree of rise or fall of minimum temperature above or below 74.2°F , between 69°F and 81°F

(3) the percentage of infection to the rainfall is $+0.1107$, i.e., for an increase or decrease of every inch of rainfall per week above or below 4.8 inches and between 1 inch and 19 inches of rainfall, there will be an average increase or decrease of 0.1107 per cent of infection

(4) the percentage of infection to humidity, i.e., for an increase or decrease of every unit of humidity above or below 83.30 per cent, and between the limits of 73 and 89 per cent there will be an average increase or decrease of infection of 0.1341 per cent

(iii) Partial correlation coefficients

In actual practice the movements of some variable in which we are interested are affected not only by the influence of one single other variable but by the coincident movements of a number of others. In the particular investigation before us we have, as stated, examined four other variables, those of maximum temperature, minimum temperature, rainfall, and humidity, and the use of 'partial correlations' has enabled us to solve the problem of the influence of each

The calculation of the partial correlations for those factors, eliminating the remaining values, gives us a most valuable quantitative means of judging to what extent they are of importance in the development of the infection

It will be realized that the partial correlation coefficient gives us approximately the same answer as we would get if we actually calculated the coefficients within each constant group and took their average, weighted by the number of observations in each group

Below I have thus tried to elucidate the influence of each of the four factors mentioned on the percentage of infection

The coefficient of correlation (r) between humidity on the one hand and rainfall and minimum temperature on the other being as follows —

(5) r of percentage of humidity with rainfall $+0.3001 \pm 0.1372$

(6) „ „ „ „ „ min temp $+0.5483 \pm 0.1054$

I have found that when —

(1) rainfall is constant, correlation of infection with humidity, $+0.2455$

(2) when humidity is constant, correlation of infection with rainfall, $+0.2226$

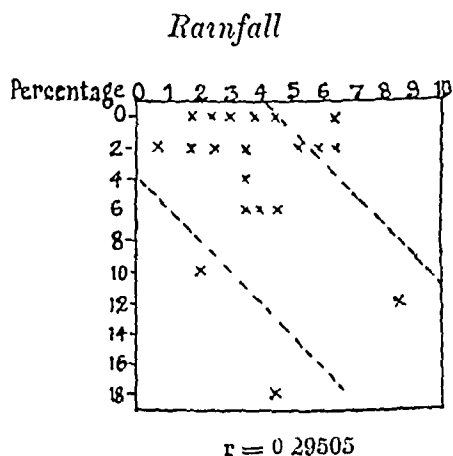
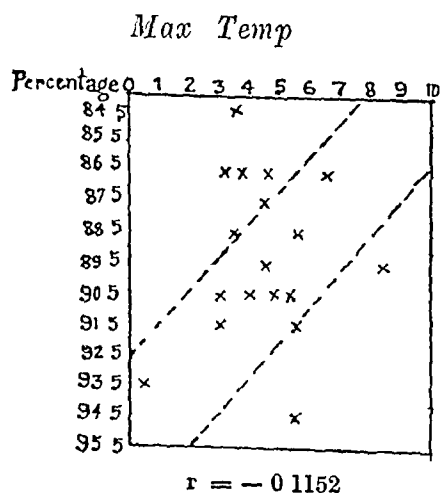
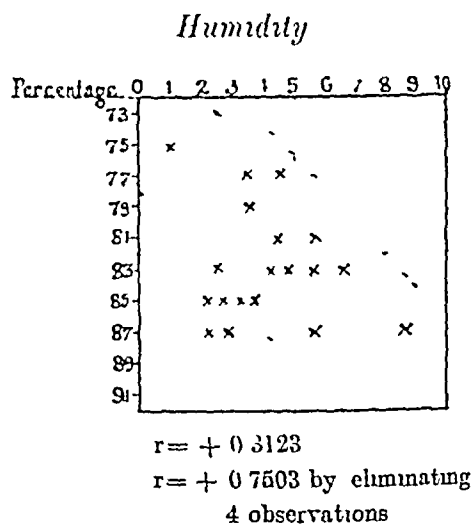
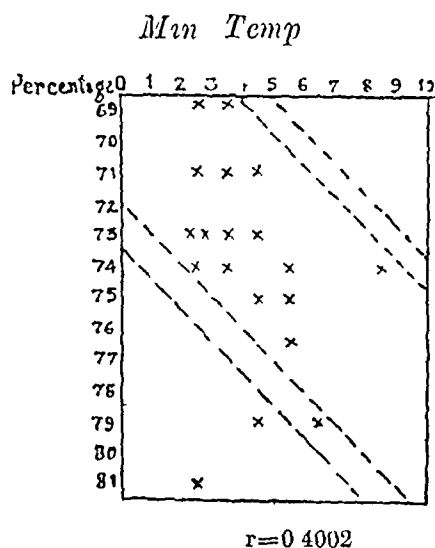
(3) when minimum temperature is constant, correlation of infection with humidity, $+0.1112$

(4) when humidity is constant, correlation of infection with minimum temperature, $+0.2786$

(iv) *Diagrammatic method*

The following Chart shows the result of this method of approaching the problem. The great significance of humidity, if one disregards four of the observational data, is shown.

CHART



The more important emergent facts of the above analysis may be here recapitulated.—

Within the limits of the observations

(1) There was a definite relationship between the malaria infection-rate of *A. funestus*, and various physical phenomena examined

(2) The relationship of the infection-rate to each factor examined was not always significant, for instance that to the maximum temperature

(3) There was a significant correlation of the infection with the humidity, minimum temperature and rainfall

(4) The minimum temperature was predominantly significant

(5) We may conclude that all the factors combined play a great part in determining the infection

(6) Their positive importance in order was —

Minimum temperature

Humidity

Rainfall

Thus, the methods employed in analysing the data have informed us to what extent the percentage of infection was influenced by the various meteorological factors

STUDIES ON THE ANTIGENIC STRUCTURE OF *VIBRIO CHOLERÆ*

Part II.

ANALYSIS OF THE CARBOHYDRATES OF PATHOGENIC AND NON-PATHOGENIC VIBRIOS

BY

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STUDIES of bacterial carbohydrates made in the last decade have given bacteriologists an entirely new basis from which to consider questions of bacterial virulence, antigenic power, and type specificity. The results of much of this work have been reviewed by Heidelberger (1927) and Cole (1931) and need not be discussed here. It seemed of interest to apply to the vibrios the methods which have proved so fruitful in the study of other organisms. Our work represents an attempt to determine the relationship, from the point of view of carbohydrate specificity, between pathogenic and non-pathogenic vibrios, smooth and rough vibrios, and strains resistant to various cholera bacteriophages.

Essentially, the method used has been to separate the carbohydrate fractions from the organisms and to study their serological and chemical properties.

By the fractionation of the vibrios, we have been able to show (Linton, 1932) that all of them contain a very similar carbohydrate group. This statement applies not only to the pathogenic vibrios, but equally to water-vibrios, smooth and rough vibrios, and agglutinating and non-agglutinating vibrios. Each of them possesses, in its carbohydrate fraction, a factor which gives a precipitin reaction with antisera to any of the others. It was concluded that the vibrios from the sources studied form a closely related group, the members of which are characterized by the possession of a carbohydrate fraction so similar in all that it cross-reacted throughout

But while it was clear from this work that all the vibrios were very closely allied it was not possible to say to what degree this similarity extended. A further investigation was necessary before any conclusion could be drawn, or any attempt made to group the vibrios into types on the basis of the constitution of their specific substances.

This further investigation, which has involved the chemical analysis of the carbohydrates as well as a comparative study of carbohydrates from different vibrios, has now been made and the object of this paper is to present these findings in a qualitative way.

ORGANISMS USED

Carbohydrates have been extracted and analysed from the following organisms —

1 *Vibrio* 454, *smooth* — A typical pathogenic *Vibrio cholerae* recently received from the Bengal Vaccine Laboratory, Entally, through the courtesy of Dr Basu. This strain was at that time among those being used in the preparation of vaccine. It agglutinated in high dilution, and was strongly agglutinogenic.

2 *Vibrio* 454, *rough* — This strain was derived from the first by the action of cholera phage A. The type of growth was pronouncedly rough, and the organism itself had become non-agglutinable as a result of the cholera phage action. We are indebted to Captain C. L. Pasucha, I.M.S. for the cholera phage treatment, and for determining the subsequent agglutination reaction.

3 WS41 — A water-vibrio, smooth, and non-agglutinable.

4 *Vibrio E* — A smooth-rough vibrio agglutinable at 1:500.

METHODS USED FOR PREPARING THE SPECIFIC CARBOHYDRATES

Roux flasks of 1 litre capacity were prepared with agar of pH 7.8 and papain digest broth and sown with the vibrios. Forty-eight hours later the thick growth was washed off with normal saline, 10 c.c. of which were used for each bottle. Two hundred to 300 flasks were worked with as a unit, as it was only by large scale methods that adequate amounts of the substance could be obtained. Glacial acetic acid was added to the washings to make a N/20 solution, and the organisms were then boiled under reflux until they coagulated. In coagulation the organisms form heavy clumps which fall to the bottom of the flask as the solution cools, and leave behind a yellow-brown syrupy fluid which is perfectly clear and which contains the specific substance. Coagulation has been taken as an indication that the specific substance is separated from the organisms. With rough strains coagulation may occur as soon as boiling begins, or after heating for periods up to one hour. Smooth strains have in general longer periods which may extend from three to six hours.

The extract and the organisms are put through a Sharples supercentrifuge at approximately 30,000 r.p.m., and the resulting clear fluid further freed from bacteria and bacterial debris by passing it while hot through a Sertiz filter. The final fluid is water-clear and of a golden brown colour. It is concentrated from its volume of two or three litres to 500 c.c. on the water-bath, cooled, and precipitated with three volumes of absolute alcohol in tall cylinders.

After standing overnight in the refrigerator the supernatant alcohol was poured off, and the heavy flocculent precipitate was taken up in water and stirred and shaken until it was dissolved. A few cubic centimetres of glacial acetic acid were usually added to facilitate solution. The solution was then concentrated on the water-bath, usually to 500 c c, and reprecipitated, and this process was repeated until six precipitations in all had been made. A larger number of precipitations was inadvisable because the amount of further purification obtained was more than offset by the loss of material in the manipulations. After the first or second precipitations the volume of the water-solution was usually concentrated to 250 c c, and the gum precipitated with three volumes of alcohol in a litre cylinder. The product of the final precipitation was a spongy mass, light gray to white in colour, and filling space to the 100 c c mark in a litre cylinder. It gives negative reactions when subjected to the usual protein tests (biuret, xanthoproteic, Hopkins-Cole, and Millon's), and it gives a strong Molisch test when its solution is diluted as much as 1 : 1,000,000. After drying *in vacuo* it forms a light brown brittle gum.

METHODS AND RESULTS OF ANALYSIS OF THE SPECIFIC CARBOHYDRATES

Carbohydrate from vibrio 454, smooth—Twenty-five c c of 50 per cent H_2SO_4 were added to 10 grammes of the gum. After keeping in the ice-box overnight the mixture was dissolved in water and made up to 500 c c, thus bringing the concentration of the acid to about N/1. After boiling on the sand-bath under reflux for two or three hours the amount of reducing substance was determined and the boiling then continued until the amount of reducing substance ceased to increase. At the point where this occurred the amount of reducing substance was usually 35 to 40 per cent, calculated as glucose, of the original gum. The acid was quantitatively removed by the addition of barium oxide, both powdered and in solution, and the resulting barium sulphate was filtered off and washed with boiling water until the filtrate was free from reducing substance. The final volume of the filtrate after washing was usually about one and one-half litres. The filtrate was concentrated under reduced pressure to about 30 c c or 40 c c, and made up to 100 c c. A further estimate of the amount of reducing substance was made at this point, and in no case was a loss greater than 4 per cent found, and in most cases no loss had occurred.

A portion of the hydrolysate was decolorized with charcoal, and a phenylosazone formed from the clear fluid. The osazone crystals were divisible into two parts. The first, which was soluble in boiling water, after re-crystallization had a melting point of 178°C to 184°C. The second portion, which was insoluble in boiling water, was washed in absolute methyl alcohol and had a melting point of 198°C to 200°C. The presence of two reducing substances in the hydrolysate was thus indicated.

The hydrolysate was then separated into three fractions. In the earlier experiments, fractional precipitation by absolute alcohol was used, but as will be seen, the resulting fractions were far from being completely pure. In the later work, the formation of barium or calcium salts has been resorted to, and has resulted in more clean-cut separations. With alcohol, the first fraction appeared upon the addition of three volumes. It was a reddish-brown sticky mass which was taken off in the centrifuge (Fraction I). After centrifugalizing the supernatant fluid was

concentrated to a small volume, again precipitated with three volumes of alcohol, and Fraction I separated once more. The process was repeated four times in all. Fraction I in turn was itself dissolved in water, and precipitated with three volumes of alcohol, in order to free it as far as possible from Fractions II and III. The supernatant fluids after throwing down Fraction I were added together and a second fraction (Fraction II) was separated upon the addition of six volumes of alcohol. This precipitation was repeated once or twice, and the precipitate itself was dissolved in water and re-precipitated. The precipitate was white, flocculent and bulky. The supernatant fluid of Fraction II was designated Fraction III.

The experimental work on these fractions may be briefly outlined —

Fraction I — This fraction did not show any reducing power even after prolonged hydrolysis with N/1 sulphuric acid. One sample was boiled for eight hours on the sand-bath under reflux, and then autoclaved for 45 minutes at 15 lb pressure without any reducing substance appearing in it.

Fraction II — The precipitate, after drying *in vacuo*, was a white granular mass easily powdered, and quite soluble in water. It was taken up in N/1 sulphuric acid and hydrolysed further, in some cases in the autoclave, and in others on the sand bath for prolonged periods. In various experiments this treatment increased the amounts of reducing substance by 25 to 50 per cent of that originally present. It is to be noted that these increases took place after the whole carbohydrate had been heated for a length of time such that its hydrolysis had ceased, or until the reducing power of the hydrolysate was beginning to decrease. The further hydrolysis of Fraction II shows that it was a complex substance difficultly hydrolysable, and the constituent parts of which have a greater reducing power than their combination. The hydrolysate of Fraction II was freed from sulphuric acid as before, concentrated and the residue taken up in water, and nitric acid added to make a strength of 60 per cent. This solution was evaporated to about one-third of its original volume on the water-bath and then set aside until the next day, when about half the volume of water was added. Crystals of mucic acid appeared within a few hours, and after standing for 24 hours longer they were filtered off, washed with water and dried. Melting point 212°C . Other samples gave melting points of 212°C to 217°C , and 212°C to 215°C . The acid bath had a temperature of about 200°C when the melting point tube was inserted. The filtrate from the mucic acid crystals was evaporated to dryness with constant stirring, to remove all the nitric acid. The residue was taken up in a small quantity of water, made alkaline with potassium carbonate, and then strongly acidified with glacial acetic acid. The final solution was concentrated somewhat and set aside. In 24 hours a good yield of the characteristic crystals of potassium acid saccharate was obtained. The finding of mucic acid in the hydrolysate of Fraction II points definitely to the presence of galactose. Potassium acid saccharate may result either from glyconic acid or from glucose. Our reasons for supposing that the first of these is the one actually present will be given below. This fraction gave a strong furfural test.

Fraction III — After evaporating the fraction to dryness it was taken up in water, decolorized with charcoal, and the following compounds prepared —

(1) Phenyllosazone, melting point, 186°C . The melting point of galactose-phenyllosazone is 188°C (Zemplén, 1922). A sample of the phenyllosazone washed in

concentrated to a small volume, again precipitated with three volumes of alcohol, and Fraction I separated once more. The process was repeated four times in all. Fraction I in turn was itself dissolved in water and precipitated with three volumes of alcohol, in order to free it as far as possible from Fractions II and III. The supernatant fluids after throwing down Fraction I were added together and a second fraction (Fraction II) was separated upon the addition of six volumes of alcohol. This precipitation was repeated once or twice, and the precipitate itself was dissolved in water and re-precipitated. The precipitate was white, flocculent and bulky. The supernatant fluid of Fraction II was designated Fraction III.

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Fraction I — This fraction did not show any reducing power even after prolonged hydrolysis with N/1 sulphuric acid. One sample was boiled for eight hours on the sand-bath under reflux, and then autoclaved for 15 minutes at 15 lb pressure, without any reducing substance appearing in it.

Fraction II — The precipitate, after drying *in vacuo*, was a white granular mass easily powdered, and quite soluble in water. It was taken up in N/1 sulphuric acid and hydrolysed further, in some cases in the autoclave, and in others on the sand bath for prolonged periods. In various experiments this treatment increased the amounts of reducing substance by 25 to 50 per cent of that originally present. It is to be noted that these increases took place after the whole carbohydrate had been heated for a length of time such that its hydrolysis had ceased, or until the reducing power of the hydrolysate was beginning to decrease. The further hydrolysis of Fraction II shows that it was a complex substance difficultly hydrolysable, and the constituent parts of which have a greater reducing power than their combination. The hydrolysate of Fraction II was freed from sulphuric acid as before, concentrated, and the residue taken up in water, and nitric acid added to make a strength of 60 per cent. This solution was evaporated to about one-third of its original volume on the water-bath and then set aside until the next day, when about half the volume of water was added. Crystals of mucic acid appeared within a few hours, and after standing for 24 hours longer they were filtered off, washed with water and dried. Melting point 212°C . Other samples gave melting points of 212°C to 217°C , and 212°C to 215°C . The acid bath had a temperature of about 200°C when the melting point tube was inserted. The filtrate from the mucic acid crystals was evaporated to dryness with constant stirring, to remove all the nitric acid. The residue was taken up in a small quantity of water, made alkaline with potassium carbonate, and then strongly acidified with glacial acetic acid. The final solution was concentrated somewhat and set aside. In 24 hours a good yield of the characteristic crystals of potassium acid saccharate was obtained. The finding of mucic acid in the hydrolysate of Fraction II points definitely to the presence of galactose. Potassium acid saccharate may result either from glyconic acid or from glucose. Our reasons for supposing that the first of these is the one actually present will be given below. This fraction gave a strong furfural test.

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Carbohydrate from water-ubino 811, smooth —The gum was treated with sulphuric acid as in the previous experiments but instead of boiling on the sand-bath, the mixture was placed for 18 hours in the incubator at 37°C. It was then heated for two hours at 80°C to 90°C. This mild hydrolysis was resorted to in an attempt to minimize the hydrolysis of Fraction II, and thus to lessen the amount of destruction undergone by its supposed glucuronic acid constituent, which has been shown to be sensitive to heat (Heidelberger and Kendall, 1929). The reducing power of the hydrolysate was 26 per cent, calculated as glucose. A portion of the hydrolysate was decolorized and the phenylosazone formed. As in previous experiments, two types of crystals were obtained. The first, which was soluble in boiling water, melted at 163°C to 165°C and a second portion, insoluble in boiling water, melted at 198°C. The presence of two substances in the hydrolysate was thus indicated.

Fraction II separated as the calcium salt —The hydrolysate was neutralized with extra-pure CaCO_3 and the calcium sulphate filtered off and washed free from reducing substances. The filtrate was decolorized with charcoal, and the colourless solution heated at a low temperature with excess of CaCO_3 . The whole solution was evaporated to a small volume and filtered. After further concentration of the filtrate, it was added with constant stirring to four volumes of absolute alcohol. The light coloured flocculent precipitate was dissolved in water, re-precipitated, and dried *in vacuo*. The filtrate, which contained Fraction III, was concentrated and re-precipitated to get rid of any remainder of the calcium salt.

The calcium salt, which is presumably a calcium aldobionate, was decomposed with sulphuric acid, and autoclaved for one hour at 120°C. The reducing substances were increased 50 per cent by this treatment. This hydrolysate also yielded mucic acid and potassium acid saccharate.

Fraction II was also separated by alcohol, by the method previously described. Upon further heating (one hour and 15 minutes at 120°C in the autoclave) it increased 33 per cent in reducing power. This portion also yielded mucic acid and potassium acid saccharate.

Fraction III, which remained after the removal of Fraction II as the calcium salt, was evaporated to dryness, taken up in a little water and the following compounds formed —

(1) Phenylosazone. M.p. 157°C to 160°C. A sample of pure arabinose-phenylosazone melted at 158°C.

(2) Methylphenylhydrozone. M.p. 163°C and 164°C. Known arabinose-methylphenylhydrozone melts at 164°C (Zemplén, 1922).

(3) *p*-bromphenylhydrozone. One part of *p*-bromphenylhydrazine, together with 3.5 parts of 50 per cent acetic acid and 12 parts of water, was mixed with an amount of Fraction III calculated to contain 0.5 parts of the reducing substance. Crystallization began after some time at room temperature, and after keeping overnight the crystals were filtered off. Owing to the smallness of the yield it was impossible to purify the crystals. They melted from 155°C to 160°C. Known arabinose-*p*-bromphenylhydrozone melts at 162°C.

(4) Mucic acid and potassium acid saccharate were formed from this fraction. The mucic acid melted at 217°C to 218°C.

The furfural test was positive.

J, MR

when separated as the calcium salt, shows on hydrolysis only galactose and glycuronic acid

We are now in a position to give a tentative answer to the question with which this part of the work began. Although the specific factors of the vibrios are so closely allied that they cross-react serologically in high dilution, yet the specific substances themselves are not identical and therefore the vibrios from which they are derived are different. Our work so far has shown that the vibrios studied fall into two groups—those containing galactose and those having arabinose in their specific substances. It appears that at least one of the chemical differences between a pathogenic vibrio and a water-vibrio lies in the possession of galactose by the first and of arabinose by the second.

Two further points may be briefly mentioned. The smooth-rough transition, brought about by bacteriophage action, does not alter the character of the carbohydrate, nor cause any change in its amount. Vibrio thus differs from pneumococcus, in which roughness is correlated with almost complete disappearance of the specific substance. In the second place, the carbohydrate does not vary in composition or amount with the agglutination reaction. The constitution of the specific substance was the same in the smooth agglutinating strain as in the rough non-agglutinating strain derived from it, and the weakly agglutinating E strain had the same carbohydrate structure as the non-agglutinating water-vibrio.

SUMMARY

Specific carbohydrates extracted from pathogenic and water-vibrios have been analysed. The identification of their constituents has led to the hypothesis that the carbohydrate of each vibrio contains an aldobionic acid made up of galactose and glycuronic acid, that a second sugar is also present, and that in some vibrios this sugar is galactose, while in other forms it is arabinose.

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OVIPOSITION IN MOSQUITOES OF THE SUB-GENUS *MANSONIOIDES*

BY

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[Received for publication, January 24, 1933]

THE process of oviposition in mosquitoes of the sub-genus *Mansonioides* (Genus *Mansonia*) shows an interesting divergence from those observed among other mosquitoes, and it would appear that this peculiar oviposition in *Mansonioides* has not been described previously. While other mosquitoes lay eggs singly or in rafts on the surface of water, *Mansonioides* lay their eggs in clusters cemented on to leaves of the floating water-weed *Pistia stratiotes*, generally on the lower side of leaves touching the water-surface. Such oviposition has been observed in all the three species of *Mansonioides* studied, namely, *M. annuliferus* Theob, *M. uniformis* Theob, and *M. indiana* Edw.

Egg-clusters of *Mansonioides* are black, usually circular in outline and consist of numerous eggs cemented together and fixed on to the *Pistia* leaf. Plate III, fig 1 is a photograph of the lower side of a leaf of *Pistia* showing six egg-clusters close to the edge of the leaf. In nature, these egg-clusters are commonly observed attached to leaves of *Pistia* in ponds in which *Mansonioides* larvæ occur. Egg-clusters of *Mansonioides* could also be obtained by enclosing gravid female mosquitoes in jars containing a fresh *Pistia* plant in water (Plate III, figs 2 and 3).

A close examination of the egg-cluster shows the pointed apices of the eggs sticking out of the cluster like the quills of a porcupine. The egg-clusters are 1.5 mm to 2.5 mm in diameter and consist of 75 to 120 eggs. No constant differences have been observed either in the size of the egg-clusters or in the number of eggs that constitute the cluster in the three species of *Mansonioides*. Specimens collected in nature, as well as those obtained from gravid females made to oviposit in the laboratory, show that egg-clusters are usually laid close to the lateral or apical edge of the lower side of *Pistia* leaves that touch the water-surface. Rarely egg-clusters occur on the upper side of the leaf in which case they are generally confined to the basal area that is submerged in water. For egg-laying, *Mansonioides* usually selects *Pistia* leaves touching the water-surface. In leaves of *Pistia* that stand out at an angle to the water-surface, egg-masses may be laid on the portion immediately below the water-surface. In all cases observed, both in nature and in the laboratory, egg-masses occur on submerged portions of *Pistia* leaves, one to two millimetres below the water-surface.

When gravid *Mansonioides* are enclosed in jars with *Lemna polyrrhiza* in the absence of *Pistia*, egg-masses may be laid on the lower side of the large fronds of

this plant Several such egg-masses have been obtained on *Lemna* in this manner, but in nature egg-masses of *Mansonoides* have not been observed in any situation other than on *Pistia* leaves

Process of oviposition

The process of oviposition in *Mansonoides* is interesting and probably unique among mosquitoes since the eggs are actually laid under water The ovipositing female sits near the edge of the leaf which is touching the water-surface, with her fore- and mid-legs on the water and the hind-legs on the leaf She then dips the abdomen into the water and by flexion the tip of the abdomen is applied to the lower side of the leaf as shown in Plate IV, fig 1 In that position, the wings, which are folded up, rest on the upper side of the leaf and the edge of the leaf is wedged in between the wing and the abdomen which is almost entirely submerged in water The eggs are then extruded one by one and the base of the egg as it emerges from the abdomen is fixed on to the leaf After fixing the base of the egg to the leaf, the egg is pushed against other eggs already laid, to which it sticks, resulting in a compact cluster The result is a mass of eggs the bases of which are cemented on to the leaf surface and the sides of the eggs cemented on to the neighbouring ones in the cluster

The process described above is the usual one observed in *Mansonoides* in the act of oviposition When suitable leaves touching the water-surface are not available, she may lay eggs on leaves standing out at an angle to the water-surface In that case, the female mosquito takes a somewhat different posture as illustrated in Plate IV, fig 2 The mosquito sits on the water with her fore- and mid-legs on the water-surface and the hind-legs placed vertically on the portion of the leaf above water The wings are unfolded and pressed against the leaf and the abdomen is dipped into the water as shown in the sketch Eggs are then laid as previously described

Sections of egg-clusters show the disposition of the eggs constituting the cluster The conical base of the egg is attached to the leaf and the straight edges of the basal half of the egg are cemented on to other eggs similarly placed The tapering narrow ends of the eggs are free Plate III, figs 4 and 5 are photomicrographs of sections of egg-clusters of *Mansonoides* on *Pistia* leaves

The eggs at the centre of the cluster are placed vertically to the leaf-surface, while those at the periphery are at an angle as seen in the photomicrographs

The egg-clusters being in contact with water the larva escapes into the water when the egg hatches and directly attaches itself to the roots of the *Pistia* plant The hatching of the egg is by a sort of a circular dehiscence Plate III, fig 6 is a photomicrograph of a vertical section of an egg-cluster in which the eggs have hatched out, leaving the empty egg-capsules consisting of the basal halves of the eggs still attached to the *Pistia* leaf Plate III, fig 7 is a photomicrograph of the apical portion of the egg which separates off when the egg hatches

The writer is much indebted to Mr M A Unnikrishna Menon Field Assistant, Section of Medical Entomology, for carrying out observation on the egg-laying of *Mansonoides*, and for the two excellent sketches (Plate IV, figs 1 and 2) which illustrate the process of oviposition

PLATE III



1



4



2



5



3



7



6

- Fig 1 Photograph of the lower side of a leaf of *Pistia* showing six egg clusters of *Mansonioidea* $\times 9/10$
 Figs 2 and 3 Photomicrographs of egg clusters of *M. annuliferus* and *M. uniformis* $\times 28$
 „ 4 and 5 Vertical sections of egg clusters attached to *Pistia* leaf $\times 45$
 Fig 6 Vertical section of egg cluster after hatching $\times 45$
 „ 7 Apical portion of the egg which separates from the egg when it hatches, showing circular dehiscence $\times 31$

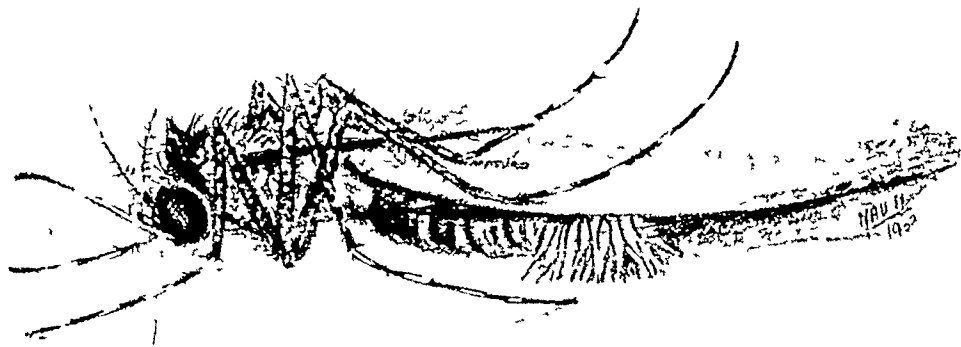


Fig 1 Sketch showing female *Mansonoides annuliferus* in the act of laying eggs on a *Pistia* leaf touching the water surface

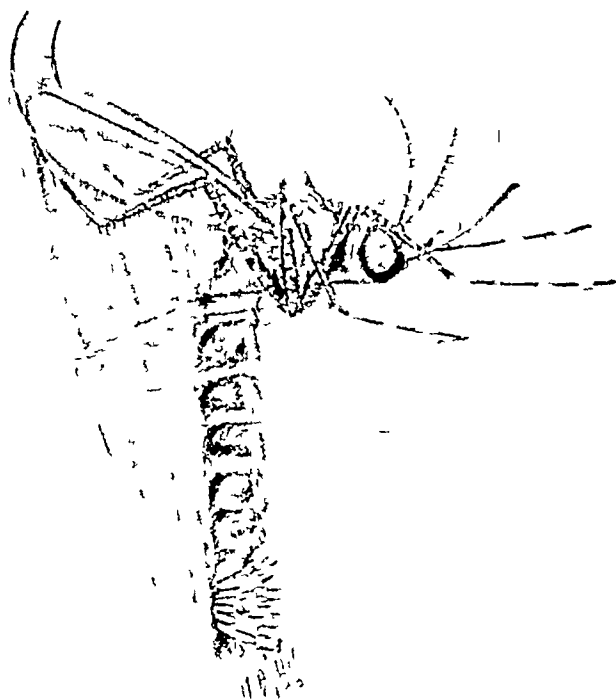


Fig 2 Sketch showing same while laying eggs on *Pistia* leaf standing out at an angle from the water surface

THE VITAMIN B CONTENT OF DIFFERENT SAMPLES OF INDIAN RICE BY SPRUYT'S COLORIMETRIC METHOD

Part II

BY

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[Received for publication, February 10, 1933]

IN our previous paper (Ghosh and Dutt, 1933) we had reported the results of our assays of several samples of rice collected in or near Calcutta either from rice dealers or from some rice mills. The present paper deals with the assay of samples of rice collected and prepared under standard conditions.

Samples of paddy with white (silver-skin) and red pericarp were secured from the Government Agricultural Department, Bengal. A relatively small portion of each sample was given to a villager to prepare 'dhenki' (hand-hulled) rice. Each sample was boiled in the usual earthen pots and then hulled by hand in a 'dhenki'. The major portion of the paddy was sent to the Ballygunge Rice Mills, Ltd. We are extremely grateful to the Managing Director, Mr C Gregory, for taking so much trouble in preparing the different samples free of cost for these experiments. Each of the two varieties of paddy was divided into two portions, one portion being dried in the sun for the 'atap' rice and the other steamed in the mill for the 'parboiled' sample. They were then hulled in the mill and samples of the 1st, 2nd and 3rd hulling collected separately and sent to us.

Six of the samples thus collected (2 'dhenki' and 4 mill) were *polished* in the laboratory. The sample of rice was taken in a cloth bag and rubbed with french chalk for several hours. Four of the samples (all mill) were also *washed* in running water for 48 hours.

Altogether 28 samples were thus obtained. They were all tested by Spruyt's colorimetric method with the modifications described in our previous paper. Each sample was examined in duplicate and the mean value of the two assays has been shown in the table.

A rough estimate of the relative proportions of husk and polishings in the paddies with white and red pericarp was also made. The rice was carefully separated from the husk and weighed before and after polishing. The red paddy showed 23.93 per cent of husk and 3.53 per cent of polishings. The white paddy showed 24.12 per cent of husk and 4.0 per cent of polishings. The percentage of polishings calculated on rice showed 5.29 in white and 4.63 in red rice.

The polishings obtained from 'atap' as well as from parboiled rice were also examined by the colorimetric method. One gramme of polishings was mixed with 9 grammes of polished and washed rice of known colour-index and the colour-value of the polishing was calculated from the final result.

The feeding experiments were carried out with munia (*Munia maja* and *Munia leucogastrides*) birds available in the local market. They were small birds of the size of a sparrow with red beak and greyish body. The diet generally given to them is 'kangni' or Italian millet (*Setaria italica*) but they ate the coarsely powdered rice grains. Six birds were kept as control on a diet of the millet and sets of six birds were given the different samples of rice. To avoid too much delay, the twice-hulled samples were used and compared with the polished and washed ones.

Several hours before death, the birds showed ruffled feathers, and shortly before death the symptoms were drowsiness, hopping with difficulty, difficulty in breathing, panting and paralysis of the head muscles, the head drooping forwards or turned backwards.

One of the dying birds was saved by feeding with a solution of vitamin B prepared more than a year ago.

TABLE I

Showing the loss in the colour-index of red and white rice during its preparation

Description of rice	Colour index	Average	Description of rice	Colour index	Average
WHITE PERICARP			WHITE PERICARP—contd		
Parboiled (milled)			'Atap' (sun dried, milled)		
Once hulled	197.11	173.81	Once hulled	191.9	150.65
Twice hulled	164.65		Twice hulled	139.2	
Thrice hulled	159.65		Thrice hulled	120.85	
Polished	121.55		Polished	78.85	
Polished and washed	31.0		Polished and washed	37.55	

TABLE I—*concd*

Description of rice	Colour index	Average	Description of rice	Colour index	Average
WHITE PERICARP— <i>concd</i> <i>Parboiled</i> ['dhenki' (hand)]			RED PERICARP— <i>concd</i> <i>'Atap'</i> (sun dried, milled)		
Once hulled	201	188.36	Once hulled	202.85	172.37
Twice hulled	188.35		Twice hulled	166.8	
Thrice hulled	175.75		Thrice hulled	147.46	
Polished	121.1		Polished	79.65	
			Polished and washed	37.05	
RED PERICARP <i>Parboiled</i> (milled)			<i>Parboiled</i> ['dhenki' (hand)]		
Once hulled	178.85	153.56	Once hulled	201.6	184.2
Twice hulled	143.7		Twice hulled	191.4	
Thrice hulled	133.15		Thrice hulled	159.8	
Polished	119.85		Polished	120.5	
Polished and washed	33.65				

TABLE II

Showing the observed and calculated colour-index of rice polishings

Polishings from	Colour index of 10 grammes of diluent rice	Observed colour index, average of 2 experiments	Calculated colour index of 10 grammes of polishings.
White parboiled rice	34.0	94.2	635
Red parboiled rice	33.6	74.35	441
White 'atap' rice	37.5	132.25	985
Red 'atap' rice	37.0	121.8	875

TABLE III

Showing the effect on muna (Munia maja and Munia leucogastroides) birds when fed on millet parboiled and sun-dried rices

Number of birds used	Food given	Dates of death	REMARKS
6	Millet	All living on the 30th day	Control
6	Milled, parboiled, white, twice hulled	Do	
6	Milled, parboiled, white, polished	Do	
6	Milled, parboiled, red, twice hulled	Do	
6	Milled, parboiled, red, polished	Do	
6	Milled 'atap' (sun dried), white, twice hulled	9th day—one dead 11th day—two dead 12th day—one dead 17th day—one dead	Total deaths 5 Average death time 12 days
6	Milled 'atap' (sun dried), white, polished	5th day—two dead 9th day—two dead 13th day—two dead	Total deaths 6 Average death time 10 days
6	Milled 'atap' (sun dried), red, twice hulled	9th day—one dead 11th day—two dead 13th day—one dead 16th day—one dead 18th day—one dead	Total deaths 6 Average death time 13 days
6	Milled 'atap' (sun dried), red, polished	10th day—three dead 11th day—one dead 13th day—two dead	Total deaths 6 Average death time 11 1 days
4	Milled 'atap' (sun dried), white, polished and washed for 48 hours	3rd day—one dead 6th day—one dead 7th day—two dead	Total deaths 4 Average death time 5 7 days

Note—It will be seen that only the birds fed on sun dried rice died

Discussion—A perusal of the above tables shows some interesting points. Firstly, the effect of hulling and polishing is quite evident. In every sample, mill or 'dhenki', white or red, parboiled or 'atap', there is always a regular fall of the colour-index with the degree of hulling and polishing. The effect of washing is most striking and there is a very big drop in the colour-index. The effect of polishing is very much more marked in the case of 'atap'.

the case of parboiled samples. The average drop in the former case is from about 161 to 79 and in the latter case from about 175 to 121. 'Atap' rice is generally brittle and its pericarp can be easily detached from the grains and thus the colour-index is likely to fall rapidly. In parboiled rice the vitamin B, being water-soluble, may partially penetrate the grains. Steaming and boiling may also make the grains more stiff on drying and the pericarp is likely to adhere to the surface more firmly. The difference in vitamin B content of 'atap' and of parboiled rice would also account for the difference in their respective polishings. One should thus expect that the polishings from 'atap' rice would be richer in vitamin B than those from parboiled rice. The experimental results corroborate this view. The feeding experiments also confirm some of the results of the colorimetric method. The birds fed on parboiled rice did not die up to the 30th day. The average death time for birds fed on 'atap' rice fell with the colour-index as shown in Table III, while the average death time of birds fed on polished and washed 'atap' showed the lowest value consistent with the very low colour-index. It will not, however, be safe to conclude from the small number of feeding experiments that there is any strict quantitative variation of the colour-index with the vitamin B content although the experiments point to some amount of correlation. If, however, we admit the correlation shown by Sprunt, the present results will enlighten us on many problems connected with Indian rice.

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SOME APPLICATIONS OF QUANTITATIVE SPECTROSCOPIC ANALYSIS

BY

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In a previous paper (Boyd and De, 1933) we have dealt with some of the aspects of qualitative spectroscopic analysis in connection with biochemical research. In this paper we shall consider this type of analysis applied quantitatively to two problems that we have been investigating, namely, the estimation of lead in urine and the estimation of manganese in certain food-stuffs and organs.

Quantitative spectrographic analysis which originated with Hartley has within recent years been developed enormously, particularly in metallurgical practice and several methods of application are now available such as the Merton Wedge method, the logarithmic sector method, and, the simplest of all, the line intensity method. It is with the latter that we shall deal with in this paper.

Line intensity method of quantitative spectral analysis

The principal of the method is based on the assumption that according to the concentration of a constituent-element in a mixture, so should the intensity of the spectral lines vary when the substance is excited in a suitable manner. If argued on a purely physical basis this assumption is not quite correct, but it has undoubtedly a great practical value. We may now proceed to describe the method as applied to the two problems noted above.

Part I.

THE QUANTITATIVE ANALYSIS OF LEAD IN URINE

We took up this problem as we had already devoted much time to it, using microchemical methods and we were thus in a position to compare results obtained by quite different methods of analysis.

DESCRIPTION OF THE METHOD

Standard plates were first prepared showing the line intensities obtained from various known amounts of lead. To prepare these we first experimented with known solutions of lead nitrate and precipitated the lead as a sulphide, but owing to the uncertainty of actually precipitating all the lead by this method we abandoned it and proceeded instead to employ known dilutions of lead acetate which were dried out direct on the water-bath, adding first a little pure sodium chloride to increase the bulk of the residue. This residue was then transferred to a suitably prepared H S brand graphite electrode, and the arc struck, using a current of four amperes at 220 volts. The time of exposure was thirty seconds. The plates used were Ilford Isozenith, speed 700. To guard against impurities in the electrodes, preliminary blank spectrographic examinations were carried out using a more powerful current and a longer exposure.

During the preparation of the standard plates we obtained the following results. The R U lines (Twyman) of lead are invariably present with 0.0001 milligram of the metal, in many cases we found the lines to persist up to 0.00001 milligram, while occasionally we have detected them in 0.000001 milligram. Plate V shows a print taken from one of these standard plates with the dilutions noted.

PREPARATION OF THE URINE FOR ANALYSIS

Two methods were employed, for very small amounts we used direct ashing but for larger amounts we acidulated the sample with acetic acid and electrolytically deposited the lead on a platinum electrode, we added one c.c. of pure glacial acetic acid per 100 c.c. of the sample and electrolysed the solution for three and half hours using a current of 0.35 amperes, the deposit on the platinum electrode was subsequently removed by treatment with pure acetic acid, and the solution so obtained was dried on the water-bath, adding a few drops of sodium chloride solution to increase the bulk. The residue was used for the analysis.

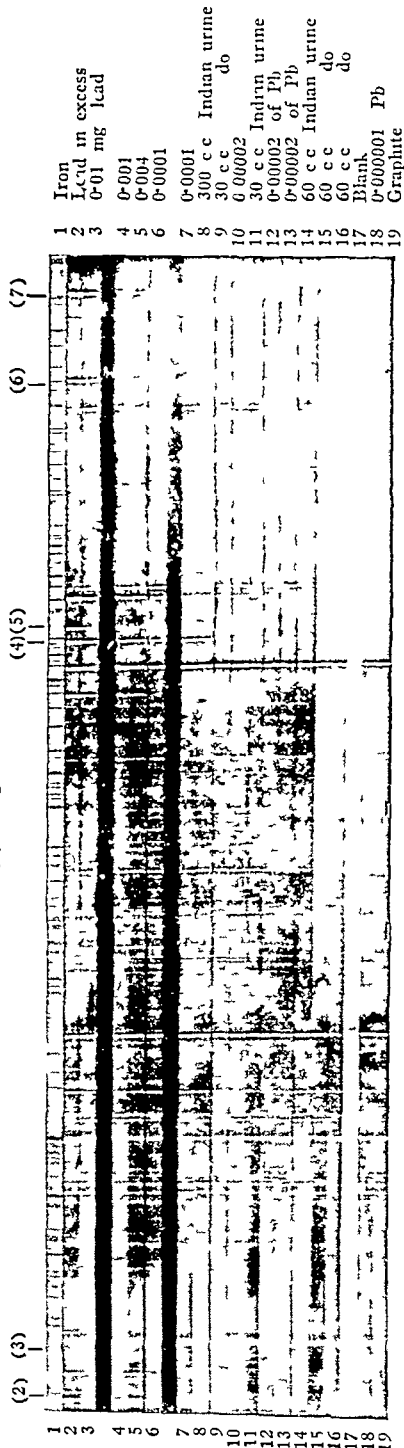
ANALYTICAL RESULTS

In a previous paper (Boyd and Ganguly, 1932) we have shown that the amount of lead present in the urine of the average European in Calcutta was far higher than that obtained from urine of Indian sources. By the spectroscopic method described above we found that we could not ordinarily detect the presence of lead in 20 c.c. of Indian urine. We frequently, however, detected it in 60 c.c. and found it invariably present when we used 300 c.c. The characteristic line 2833 Å U showing up clearly. Plate V shows some of our results and clearly demonstrates the marked difference in the intensities of the lines. Quantitative results obtained by comparing the intensities of the lines against the prepared standard plates gave the following results. The amount of lead present in 300 c.c. of Indian urine corresponds to the line on the standard plate given by 0.0001 milligram of lead while the amount found in a sample of 100 c.c. of European urine corresponded to the line produced by 0.004 milligrams of lead or 0.04 mg. per 1,000 c.c. of average European urine as against 0.0003 mg. of lead per 1,000 c.c. of average Indian urine.

PLATE V

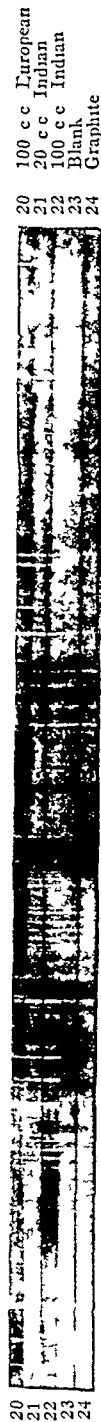
Spectra taken with various dilutions of Lead also with Electrolytical deposits from different volumes of urine

(1) Region-3700-2600 A U



Wave lengths—

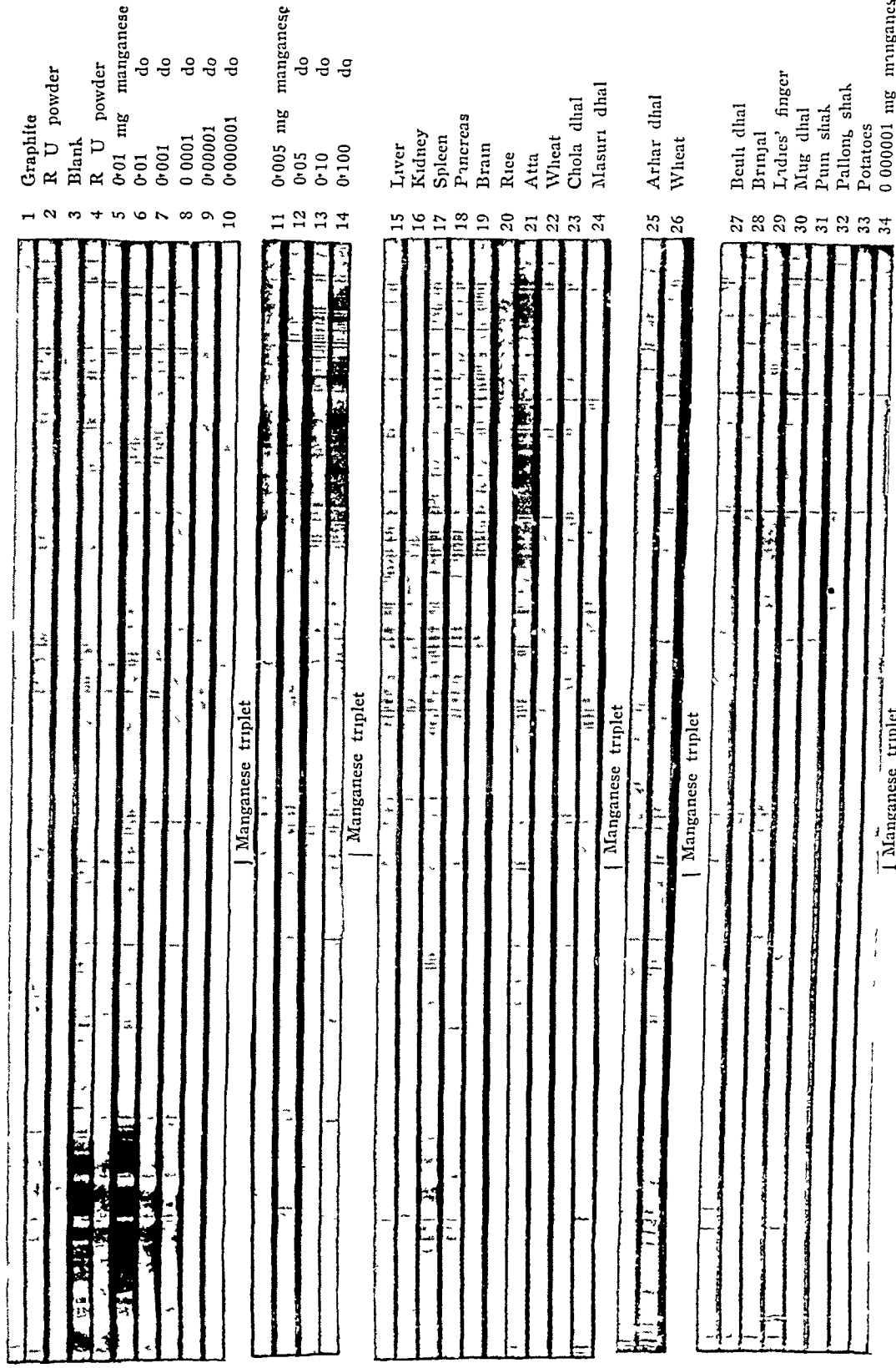
(2) 3683 47 (3) 3639 38 (3) 2833 07 (5) 2823 20 (6) 2663 (7) 2614 A U.



2833 07

In Plate V important R U lines of lead are marked and their wave lengths are noted Note the line 2833 07 This being most persistent gives the quantitative data

The plate shows spectra of some known dilutions of manganese chloride, also spectra of some important human organs and Indian food-stuffs



The manganese triplet 4034 45, 4033 07, and 4030 76 is marked in the plate. Note the spectra of wheat, atta, liver, etc., which give comparatively intense manganese lines

SUMMARY AND CONCLUSIONS

There can be little doubt that the spectroscopic method of testing urine, for the presence of lead, is extraordinarily useful, perhaps one of the drawbacks is its extreme sensitiveness. However, nowadays as the presence of lead in urine has assumed a proper perspective this fault may be dismissed. From the quantitative aspect we consider the method extremely convenient and sufficiently correct to be of the greatest practical utility, provided sufficient care is taken in avoiding possible fallacies such as may occur with impure electrodes, etc.

Part II

QUANTITATIVE ESTIMATION OF MANGANESE

The estimation of this element has recently assumed great importance in biochemical research, due to the fact that it has always been found to be present in living tissues and is particularly marked where life changes are occurring and also where vitamins are present—points brought out by Colonel McCarrison and based on analysis by Reiman, Minot, Bertrand and Rosenblatt. Wheat is particularly rich in this element, especially the outer layers. According to estimations carried out by Dr Norris the whole grain contains 4.8 milligrams per 100 grammes of the dried material. Paddy on the other hand appears to be very poor in this element.

The work of Bertrand and Rosenblatt supplied us with analytical data concerning the quantity of manganese present in some of the most important organs which we quote: Liver 0.17 milligrams per 100 grammes of fresh tissues, kidney 0.076, pancreas 0.061, muscle, brain, heart, lungs, stomach and spleen contain considerably less (from 0.014 to 0.032 mg). Blood also contains a fairly constant concentration in health. As the subject therefore appeared to be of some importance we considered that it would be suitable for the application of the spectroscopic method.

TECHNIQUE

Known weights of different substances were ashed in platinum and the total ash collected. Twenty milligrams of these ashes from the different samples were filled into the bored crater of the positive electrode (Graphite H.S. brand) and the spectrum taken by the arc method. The current used was 6.0 amperes at 220 volts and the time of exposure one minute in each case. The results obtained were compared against standard plates prepared by one of the following methods—

(1) By adding drops of known strengths of manganese chloride solutions on the electrodes.

(2) Drying out solutions of manganese chloride of known strengths with the addition of pure sodium chloride to give it the necessary bulk and placing this residue in a cored electrode.

Method (2) gave much the best results as we were able to find the manganese triplet well marked in very low dilutions (disappearing at about 0.000001 mg). The standard plates so prepared are shown in Plate VI.

The manganese triplet lines 4034.45, 4033.07 and 4030.76 Å U which are the most sensitive R U lines of the metal were used for comparison against the standard plates. The quantitative results obtained are given in the following table —

TABLE

(a) Human organs

Substance	Weight of fresh materials g	Total ash, mg	Spectral coincidence at	Manganese per 100 gs of substance, mg
Liver	66	305	0.01	0.23
Kidney	45.5	467	0.001	0.052
Pancreas	68	232	0.005	0.087
Spleen	53.4	651	0.0005	0.03
Brain	42	595	0.0002	0.014

(b) Indian food-stuffs

Substance	Weight of fresh materials, g	Total ash, mg	Spectral coincidence at	Manganese per 100 gs of substance, mg
Wheat	40	928	0.05	5.8
Atta	40	765	0.05	4.8
Rice (polished)	70	294	0.01	0.2
Chola dhal	50	761	0.01	0.76
Masuri dhal	50	700	0.01	0.70
Benli dhal	70	1,042	0.005	0.485
Mug dhal	65	1,605	0.001	0.12
Arhar dhal	50	1,210	0.0005	0.06
Potato	105	721	0.001	0.043

(c) *Indian food-stuffs*

Substance	Spectral coincidence at	Percentage of manganese in the ash
Pallong shak	0 001	0 005
Punn shak	0 001	0 005
Brinjal	0 0005	0 0025
Ladies' fingers	0 0001	0 0005

SUMMARY AND CONCLUSIONS

Parts I and II of the present paper show some practical applications of quantitative spectral analysis, which deserve consideration, particularly, when applied to biochemical problems. The method is much more rapid and searching than a detailed chemical analysis, and can moreover be applied to very small amounts of material, a point which is often of the greatest importance. As regards the question of the quantitative estimation of manganese, the contents of the various substances examined appear to agree fairly approximately with those obtained by chemical analysis where the figures are available. Spectrographically we have found that wheat and atta seem to be comparatively rich in the metal, while rice on the other hand is poor and it is of interest to speculate on Colonel McCarrison's theory that perhaps the manganese content of a diet plays an important rôle in the physical build of different nations, e.g., Sikhs as compared with Bengalis, consuming different kinds of food-stuffs.

Lastly, our thanks are due to Professor Sir C. V. Raman, F.R.S., N.L., and Dr B. Chakrabartty, Chemical Examiner to the Government of Bengal, for their interest in the work and kindly allowing us the free use of their laboratories.

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NORMAL AGE DISTRIBUTION IN INDIA.*

BY

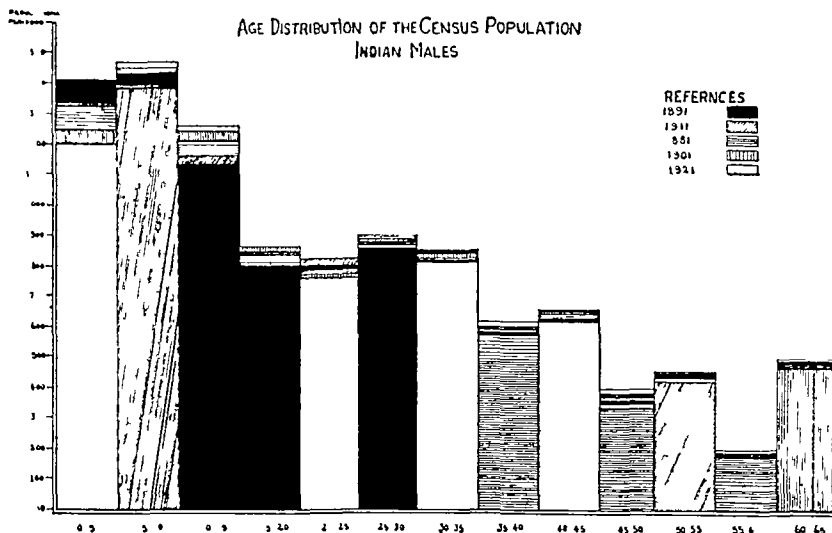
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THE age distribution in every country follows a definite tendency which practically remains unchanged (*vide* Charts I, II and III) unless disturbed by unusual happenings such as pestilence, famine, warfare, and the like The impressed

CHART I

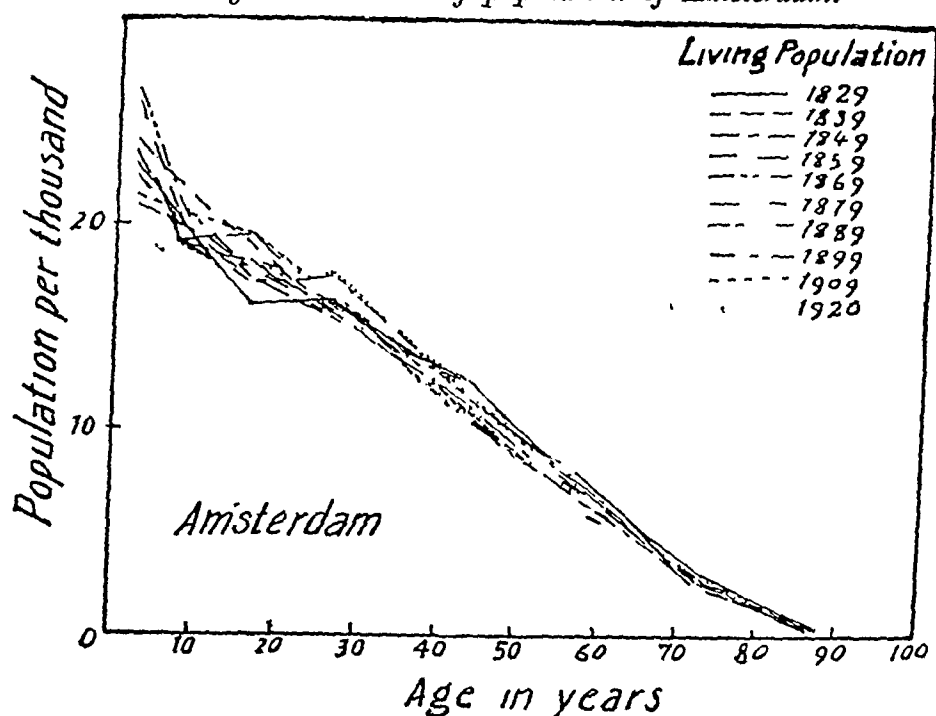


variations that result from such catastrophies persist only so long as the groups affected thereby continue to remain in existence and revert gradually to the

* Read at the Indian Science Congress, December 1932

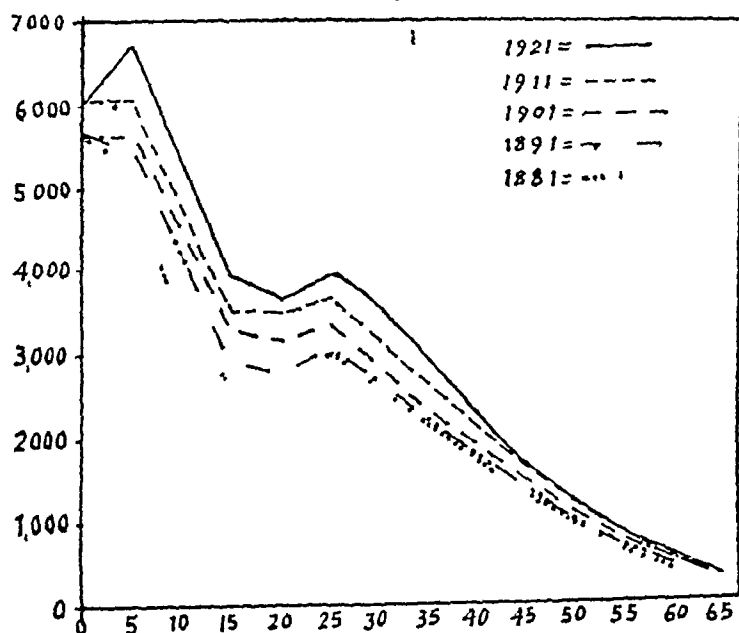
Normal Age Distribution in India.

CHART II.

Age distribution of population of Amsterdam.

(Reproduced from Pearl's 'Medical Biometry and Statistics')

CHART III.

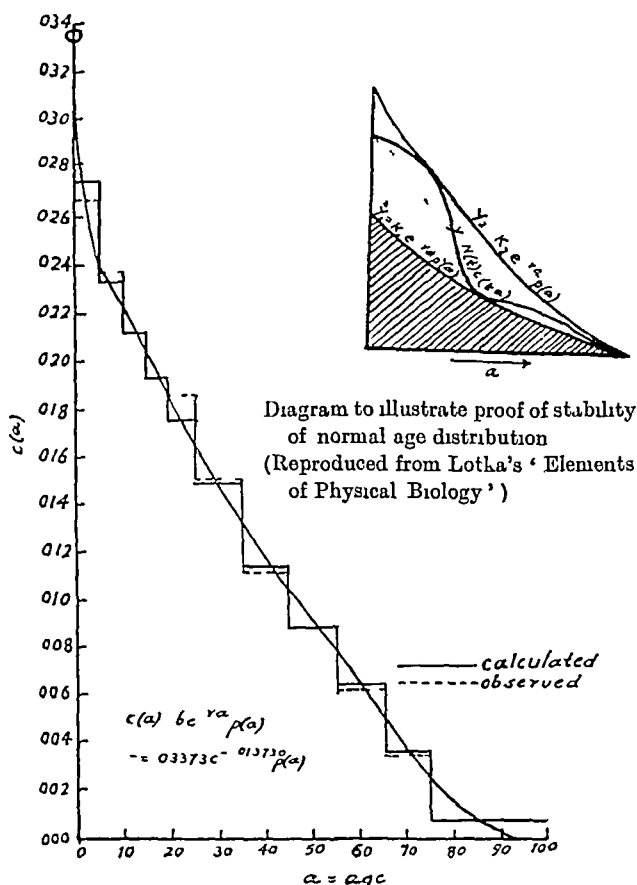
Age distribution of Bengal males

Number living between $x + x + 5$ at each census after adjustment of minor errors of age (based on Meikle's figures).

normal course as the groups begin to clear out from the stage of life. These two features of age distribution, namely, its tendency to be stable under normal conditions and its inherent inclination to revert to this condition if disturbed

CHART IV

Stable age distribution of population of England and Wales



Stable age distribution, as exemplified by the population of England and Wales in the decade 1871-1880
(Reproduced from Lotka's 'Elements of Physical Biology')

therefrom (*vide* Chart IV), have enabled us to evolve a scientific method for ascertaining the true age distribution of a given population, when only the census total is available. By elaborating this method with the help of the principle underlying the formula of Lotka regarding the relation of births to age distribution, we feel we have discovered a simple and reliable method of ascertaining

the correct age distribution in any given population The details of the method as well as the advantages of its application are dealt with below —

Basis of the formula —

Growth function $F = \frac{dx}{dt}$ = the rate of increase of the mass $x = U - V$,

when U is added to the mass per unit of time and V is eliminated therefrom per unit of time

Growth of aggregates

If N is the number of individuals and m is the average mass per head, we have total mass $x = mN$

By differentiating

$$\frac{dx}{dt} = m \frac{dN}{dt} + N \frac{dm}{dt}$$

If the average mass is constant, the second term of the right hand drops out and we have simply

$$\frac{dx}{dt} = m \frac{dN}{dt} \quad (1)$$

Now $\frac{dN}{dt}$, the rate of increase of the number in the aggregates, naturally indicates the excess of the newly formed individuals (B) per unit of time over the number (D) eliminated therefrom per unit of time, i.e., in a population of living organisms

if B —Total birth per unit of time and

D —Total deaths per unit of time, we have then

$$\frac{dN}{dt} = B - D = (b - d) N \quad (ii) \text{ when } b \text{ and } d \text{ indicate birth and death per head per unit of time}$$

Now substituting (ii) in (1)

$$\text{We have } \frac{dx}{dt} = (B - D) m \quad (iii)$$

i.e., the rate of increase of a mass x

= total addition per unit of time

— total elimination per unit of time

= total births—total deaths per unit of time

= (birth rate—death rate) \times total number

Now, if we analyse a population in a selected locality under essentially constant conditions of life and if we denote l_x , survivor to age x out of an original batch of l_0 counted at birth, we have then

$\frac{l_x}{l_0}$ called the survival factor and denoted by $p(x)$

$$\therefore l_x = l_0 p(x) \quad . \quad . \quad . \quad (iv)$$

We know the force of mortality μ_x at age x measures the death rate per head in a population composed entirely of the individuals of age x , hence

$$\frac{dl_x}{dx} = -\mu_x l_x \text{ or } -\mu_x = \frac{dl_x}{dx} \times \frac{1}{l_x} = \frac{d \log_e l_x}{dx}$$

Now the relation of $p(x)$ with μ_x —when the force of mortality is independent of age

Law of organic growth is

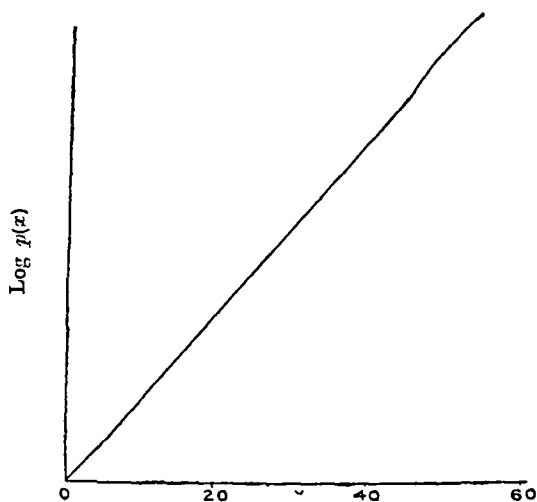
$$l_x = l_0 \times e^{-\mu x} \text{ or } e^{-\mu x} = \frac{l_x}{l_0} = p(x)$$

Taking log, $-\mu x = \log_e p(x)$

$$\text{Differentiating } \frac{d \log p(x)}{dx} = -\mu$$

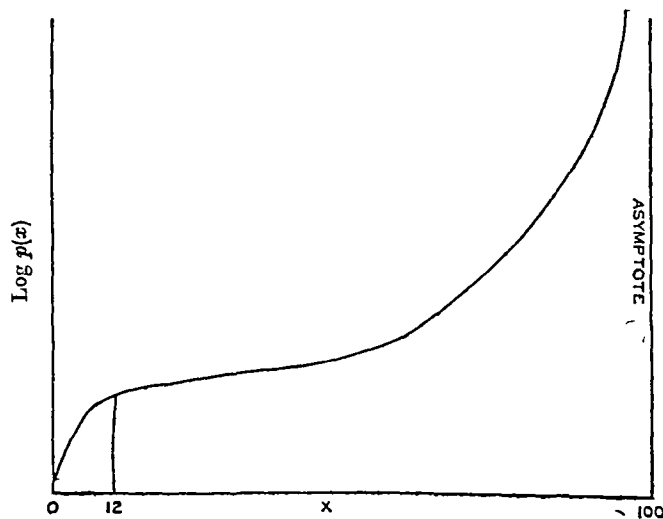
Now if we put this $p(x)$ on a logarithmic paper a straight line is obtained as shown in Diagram 1

DIAGRAM 1



$\log p(x) = -\mu x$ when μ is constant

DIAGRAM 2



$\log p(x) = -\mu x$ when μ is variable

Now as $\log p(x)$ is proportional to x , when μ is supposed to be constant, the graph showing the variation of $\log p(x)$ with x must be a straight line passing through the origin and $\frac{d \log p(x)}{dx}$ is represented in the figure by the tangent of the angle of inclination of the line to the abscissa

We know that the force of mortality is not independent of age but varies very decidedly with age. It is very high in infancy, decreases in early childhood until it reaches a minimum at about the twelfth year of life, and finally increases continually to the end of the life span.

Therefore, in the actual case of an aggregate of human population, the graph will not be a straight line but will be curved as shown in Diagram 2 with a point of inflection at $x=12$ and the line $x=100$ will be asymptotic to it.

Now what is the relation of normal age distribution to birth? According to law of organic growth $P_{t-a} = P_t e^{-ra} p(a)$ when e^{-ra} is a die-away factor.

Now births of 1921 = birth rate per head \times total population of 1921

= birth rate per head \times total population 1931 $\times e^{-ra}$

Age distribution at 10 in 1931

= total births of 1921 $\times p(10)$

= birth rate per head \times total population 1921 $\times p(10)$

= „ „ \times total population 1931 $\times e^{-r \times 10} \times p(10)$

Age distribution per head at 10 in 1931

= birth rate per head 1921 $\times e^{-r \times 10} \times p(10)$

or $c(a) = b_a \times e^{-ra} \times p(a)$ when

$c(a)$ — age distribution in time t at a age per head,

b_a — birth rate in the year $t-a$ per head,

$p(a)$ — survival factor at age a , and

r , — rate of increase per unit of time, a constant whose value is to be found out.

How can the constants be deduced?

(a) *Rate of increase r* —For example let us calculate the normal age distribution of India, assuming that the unit in which t is measured is one year and that the origin of the year is 1881. The value of r or rate of increase can be found thus, when P_t = Population in 1931 and P_{t-a} = Population in 1881 or $a = 50$.

We know $P_t = P_{t-a} e^{ra}$

$$\text{or } e^{ra} = \frac{P_t}{P_{t-a}} = \frac{P \text{ 1931}}{P \text{ 1881}}$$

$$\text{or } r = 0.002953986$$

From this the succeeding values of e^{-ra} can be easily derived when a varies (see Table VII).

Similarly, the other values of r for every ten years can be calculated by taking $a=10, 20, 30$ and so on up to 50 years, and then interpolating the values of e^{-ra} for every individual year (see Table VIII). But for periods over 50 years it will not be far from the truth if we take the rate of increase to be the same as that already worked out for 50 years.

(b) *Survival factors or $p(a)$* —Now regarding the values of $p(a)$ or survival factors, we can find them out directly from the l_x column of any standard life table, or we can make a mortality table of our own from the latest mortality figures from which we can calculate the survival factors. I have utilized the mortality

figures of Meikle (1921) which are supposed to be the latest reliable figures and have also drawn a specific death-rate curve and also a curve of survivors which are shown in Charts V and VI for the whole of India. From these I have compiled a mortality table as shown in Table IX. The l_x column of this table gives the survival factors per 10,000. As regards the figures above the age of 70 which we do not obtain from Meikle's book, I have utilized the figures of Acland (1913) in order to complete the curve.

(c) The next constant to be considered is *birth rate* 'b' per unit of head. Acland (*loc cit*) in his actuarial report on the census figures had shown that there is an omission varying between 7 and 8 per mille for birth. The result of special inquiries in Bengal suggests that about 25 per cent of deaths remain unreported and the omission in the record of births is generally between 1 and 2 per cent more than in the case of deaths. Again, while reviewing the vital statistics of the Punjab Mr Jacob states that the birth rate is 18 per cent in error. As a result of the various tests that have been made and of the general experience of those who are in the best position to estimate the value of these records, the following conclusions have been arrived at —

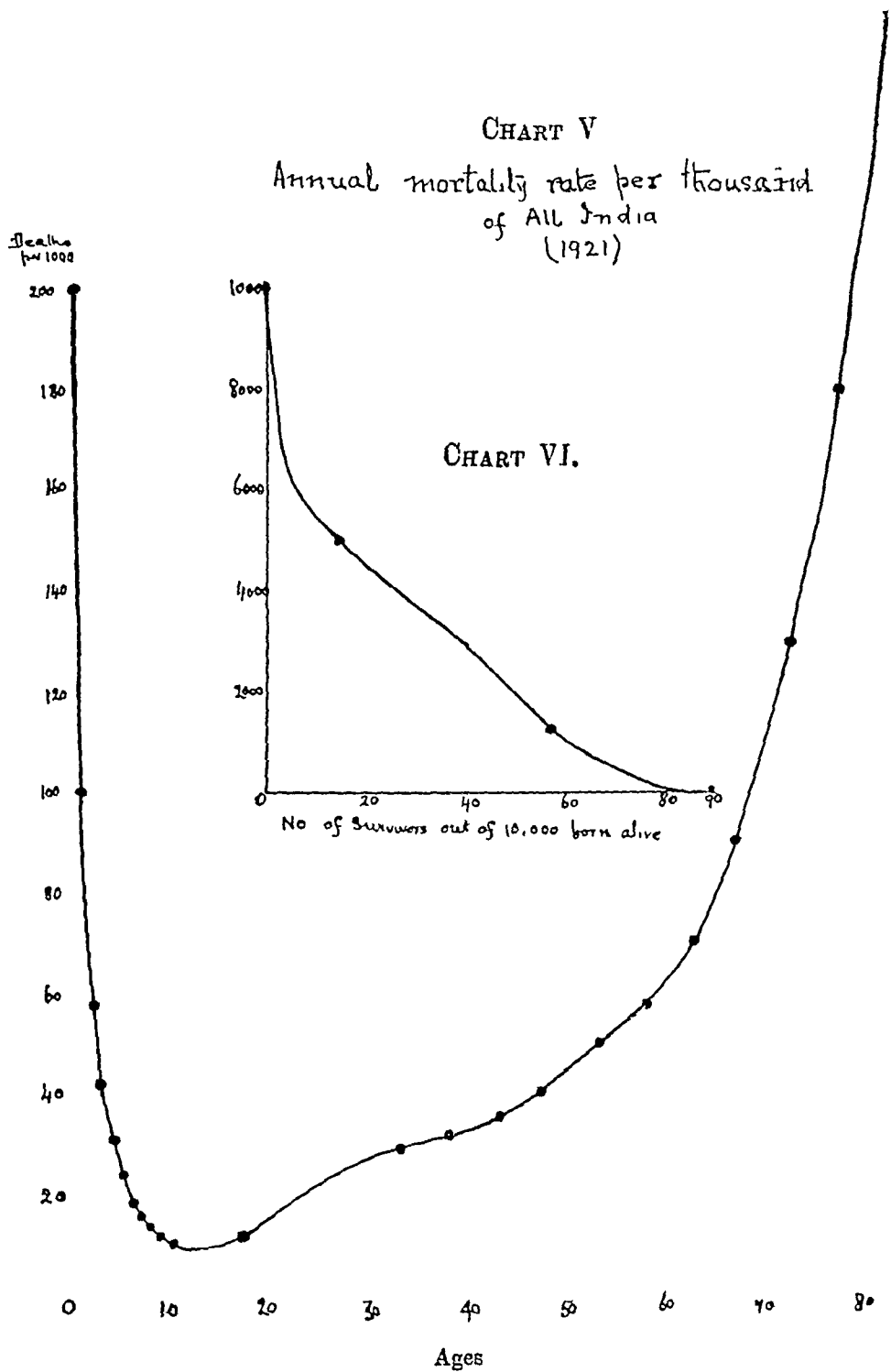
(I) Omissions in the records of the number vary up to about 20 per cent

(II) Record of births is normally less accurate than that of deaths

Analysis of the figures obtained by the special investigation undertaken by the Government of Behar and Orissa, reveals the fact that the omission in the record of birth is about 17 per cent while that of death is about 15.5 per cent (*vide* Appendix, — Actuarial Report, 1926). We all know that the natural increase of the population may be represented by the excess of births over deaths (emigrants and immigrants being excluded from the enumeration). Now from the year 1921 to 1931 birth rate in all India is 34.30 per mille, and death rate is 26.48 per mille, so the natural increase may be put down at 8.22, whereas the actual increase will come up to 9.33 per mille. Therefore the recorded number of births and deaths must have been 16.18 per cent less actually, and if we assume that the record of omission in the case of births is 1.5 per cent more than that in the case of deaths, then the actual birth rate should be 17.16 per cent more than that of the recorded rates. So the graduated figures of the recorded birth rates from the year 1900 to 1930 will, after correction, yield a curve from which the actual figures can be deduced. The figures so deduced have been found to tally with those of Hardy (1905) for the year 1901 and those of Acland for the year 1911 (Acland, 1913). The percentage of omissions in the recording of births and deaths has also been corroborated by the application of the formula on the basis of the known standardized age distributions for 1911 and 1921.

The constant v the varying rate of increase — The question of constant rate versus varying rate, though discussed in the actuarial reports, still remains an open one. Hardy (*loc cit*) adopted the constant rate of increase in calculating the age distribution in 1910, but Acland (*loc cit*) who submitted the next actuarial report adopted a different method. As regards the Punjab he worked on the basis of constant rate, while for other provinces he adopted the variable rate. Meikle (*loc cit*) has combined the two methods and accepted the mean figures resulting from the same. He has stressed the difficulties of verifying the figures in either of the methods on account of the unreliable

nature of census figures and is of opinion that the results arrived at by their combination are far more likely to be nearer the mark than those given by them separately To us it appears that there will be very little difference



in the result if either of the two methods is relied upon as a basis for calculation, but the adoption of the constant rate appears to be more desirable in view of the fact that during the period under consideration, i.e., from 1921 to 1931, factors causing fluctuation in age distribution (e.g., famine, epidemics, etc.) were practically absent. In our calculations we have taken the view that the constant rate remains practically unchanged for a decade after which allowance ought to be made for some variation in it which will continue to be the same for the next ten years. Thus the constants are always for one decade only and are assumed to vary from decade to decade, the reason for this being that the fluctuating factors like epidemics, famine, etc., are usually found confined in their effects almost to every ten years, so that some allowance for these effects appears to be necessary at the end of each decade.

Possible age distribution of census enumeration, 1931

Now as regards the possible age distribution of the census figures of 1931, this can be done in two ways, by the application of the mis-statements of age as given in Meikle's last actuarial report to 'normal' age distribution figures as calculated by our method or by the application of ratios deduced from previous census figures to the figures of 1921. We have not adopted the first method as there is no means by which the data can be checked. The second method on the other hand is a simple one and requires no verification of these data as they are all taken from the previous census reports (see Tables I and II). The method by which the ratios are obtained may be explained by means of an illustration. Suppose X represents the number of people from 0 to 5 in 1911 and X_1 of people between 10 and 15 in 1921. If we now divide the second by the first, $\frac{X_1}{X}$ will represent the ratio of the variation for the first quinary group in the second decennium. The ratios in respect of all other quinary groups in the preceding decenniums have been obtained in exactly the same way and subsequently the ratios thus obtained for each quinary group have been graduated for ascertaining the ratios for 1931 (*vide* Tables IV and V). The ratios so obtained have been applied to the age distribution figures of 1921 in order to get the possible distribution figures for 1931 (*vide* Table VI). As regards the finding out of the figures between 0 and 5 we have only to apply the method of finding out the 'normal' age distribution figures and then apply the correction factor for mis-statement of age. Lastly, we determine the quinary group for 5 to 10 by noting the balance. Reference to Tables IV and V will be found helpful in this connection.

Normal age distribution of India

Normal age distribution is generally calculated on the assumption of a fixed birth rate and a constant rate of increase. The figures are generally in five or ten year groups. In Table VIII we have worked out the normal age distribution of India (1900-1931) on this basis. We have also given in Table VII the normal age distribution for the year 1931 after allowing for the fluctuations in the birth rate as well as in the rate of increase for every ten years. It will be seen from a comparison of these tables for the first 30 years, that the figures show a marked difference which can be accounted for by the huge loss of children under 15 due to the epidemic of

influenza that prevailed at the time and by the severe reaction that it must have had on the birth rate. Another interesting fact brought out is that the distribution of 1900–1931 actually lies within the limits of the means found in Table III when studied in broader groups—such as 0–15, 15–50, and 50 up. This clearly shows that the normal age distribution follows the well-known principles of Sundberg.

Before concluding it seems desirable to say a few words by way of justifying the *correction factor* which we have adopted in the case of birth rates. The difference between the actual birth and death rates represents the natural increase in the population. If the actual increase be known then the correction factor becomes quite apparent. The figures obtained by the application of the same formula to other periods have been found to tally with the findings contained in the Government actuarial report. Moreover, the most unstable period is that between 0 and 15 and the figures for these periods, as obtained by the method explained above, being quite reliable, any subsequent application of the same for determining other figures may be safely relied upon. As regards the *survival factors* the important period is that between 0–15. This period being most unstable, the latest mortality figures have been utilized for the purpose of evolving specific death-rate curve and consequently it may be presumed that it represents perhaps the closest approximation to the true state of things.

SUMMARY AND CONCLUSION

In the foregoing pages a detailed description of a formula for determining the approximate age distribution in any population group is given. It will be evident from a study of it that when real birth rates and true survival factors are available, the actual age distribution of a population can easily be determined by the application of the formula. This formula not only enables one to predict the age distribution long before census details are available, but also helps in the checking of the correctness of census figures as well as in the obtaining of a true picture of the actual state of things. From this one ventures to presume that the formula will have a wide practical application and will be of special benefit to those who wish to make advance plans and estimates for future population groups in the matter of education, labour, town-planning, sanitary reforms, life insurance, etc.

ACKNOWLEDGMENT

My thanks are due to Lieut.-Colonel Stewart for the great help which he has given in the elaboration of the materials embodied and for the kindly encouragement bestowed on me all through.

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TABLE I
Age distribution of census reports (males)

Ages	1881	1891	1901	1911	1921
0—5	1,318	1,409	1,254	1,327	1,202
5—10	1,432	1,428	1,394	1,383	1,471
10—15	1,214	1,139	1,264	1,245	1,165
15—20	811	809	866	848	842
20—25	799	803	787	822	775
25—30	896	861	879	896	865
30—35	885	859	848	829	825
35—40	587	599	609	622	636
40—45	642	657	649	634	621
45—50	344	354	370	380	392
50—55	436	437	437	432	434
55—60	161	165	177	177	185
60—65	475	486	466	257	266
65—70				83	81
70 and over				145	160

TABLE II
Age distribution of census reports (females)

Ages	1881	1891	1901	1911	1921
0—5	1,417	1,527	1,339	1,433	1,316
5—10	1,383	1,391	1,382	1,383	1,494
10—15	1,006	946	1,082	997	1,081
15—20	729	811	835	826	815
20—25	905	897	892	930	881
25—30	925	904	895	909	885
30—35	881	846	851	835	833
35—40	527	555	557	556	565
40—45	645	626	652	631	621
45—50	218	323	339	338	346
50—55	464	426	452	444	438
55—60	157	170	169	164	168
60—65	590	573	555	305	298
65—70				78	79
70 and over				175	180

TABLE III
Analysis of census figures, 1881-1921

Ages	1881	1891	1901	1911	1921	Mean
0—15	3,964	3,976	3,912	3,875	3,918	39 29 ±0 109
15—25						
25—35	1,781	1,720	1,728	1,725	1,690	
35—50	1,573	1,610	1,628	1,636	1,649	
50 and over	1,072	1,085	1,080	1,094	1,126	10 914 ±0 106
15—50	4,964	4 942	5,009	5,031	4,956	49 80 ±0 216

TABLE IV
Ratios of $\frac{X_1}{X}$ (males)

Ages	1881-1891	1891-1901	1901-1911	1911-1921	1921-1931
0—5	0 865	0 897	0 929	0 938	0 950
5—10	0 565	0 580	0 606	0 608	0 612
10—15	0 661	0 691	0 650	0 660	0 670
15—20	1 061	1 086	1 074	1 020	1 012
20—25	1 075	1 056	1 048	1 003	1 002
25—30	0 668	0 707	0 707	0 709	0 710
30—35	0 742	0 755	0 747	0 749	0 750
35—40	0 603	0 617	0 623	0 630	0 637
40—45	0 671	0 665	0 665	0 684	0 690
45—50	0 479	0 500	0 479	0 486	0 490
50—55			0 588	0 616	0 620
55—60			0 468	0 437	0 450
60—65					
65 and over					

TABLE V
Ratio $\frac{X_1}{X}$ (females)

Ages	1881-1891	1891-1901	1901-1911	1911-1921	1921-1931
0-5	0 666	0 708	0 744	0 753	0 765
5-10	0 586	0 598	0 599	0 589	0 598
10-15	0 891	0 942	0 883	0 883	0 891
15-20	0 934	0 948	0 937	0 895	0 954
20-25	1 160	1 103	1 000	0 995	0 895
25-30	0 600	0 616	0 621	0 621	0 624
30-35	0 710	0 710	0 741	0 743	0 730
35-40	0 612	0 608	0 606	0 622	0 630
40-45	0 660	0 722	0 679	0 694	0 700
45-50	0 534	0 523	0 483	0 497	0 500
50-55				0 672	0 680
55-60				0 481	0 485
60-65				0 590	0 650
65 and over					

TABLE VI

Possible age distribution of census population, 1931

Ages	Males	Females	Total
0-5	1,277	1,377	1,327
5-10	1 487	1,490	1,489
10-15	1,142	1,007	1,075
TOTALS	3 906	3,874	3,890
15-20	846	816	833
20-25	834	942	888
25-30	859	879	869
30-35	778	786	782
35-40	615	552	583
40-45	619	622	620
45-50	405	356	381
TOTALS	4,956	4 956	4,956
50-55	428	434	431
55-60	192	176	183
60-65	269	298	284
65-70	83	82	83
70 and up	166	180	173
TOTALS	1 138	1,170	1 154
GRAND TOTALS	10 000	10,000	10,000

TABLE VII

'Normal' age distribution (1932)

Age	Birth rate b_a	After correc- tion	e^{-ra}	$p(a)$	Estimated
0	36 78	43 03	1 00000	1 0000	4,303
1	36 50	42 70	0 99034	0 7390	3,125
2	35 47	41 50	0 98077	0 6797	2,766
3	36 78	43 03	0 97130	0 6391	2,671
4	35 27	41 26	0 96192	0 6122	2,430
5	34 77	40 68	0 95263	0 5926	2,296
6	33 65	39 37	0 94342	0 5784	2,148
7	34 45	40 30	0 93432	0 5674	2,136
8	35 06	41 02	0 92529	0 5577	2,117
9	31 85	37 26	0 91635	0 5493	1,875
10	31 97	37 40	0 91136	0 5483	1,868
11	32 98	38 58	0 90642	0 5468	1,908
12	30 24	35 38	0 90149	0 5456	7,766
13	35 35	41 36	0 89659	0 5445	2,019
14	39 33	46 01	0 89172	0 5432	2,228
15	37 13	43 44	0 88637	0 5417	2,087
16	37 82	44 25	0 88205	0 5394	2,066
17	39 61	46 34	0 87725	0 5371	2,183
18	39 31	45 99	0 87249	0 5345	1,845
19	38 95	45 77	0 86735	0 5314	2,109
20	38 58	45 15	0 86224	0 5279	2,054
21	39 52	46 23	0 85716	0 5241	2,068
22	36 65	42 88	0 85211	0 5202	1,889
23	37 78	44 20	0 84709	0 5163	1,921
24	37 86	44 22	0 84210	0 5123	1,881
25	39 13	45 78	0 83914	0 5082	2,024
26	40 86	47 80	0 83221	0 5040	1,993
27	38 96	45 58	0 82730	0 5010	1,878
28	39 38	46 07	0 82243	0 4968	1,871
29	34 67	40 56	0 81758	0 4925	1,623
30—35		42 80			

TABLE VIII

Normal age distribution of Indra (1900-1931)

	b_a	\times	est	$\times p(a)$	Population per 100,000	
0-1	43	\times	1 00000	\times 1 0000	4,300	
1-2	"		0 99705	\times 0 7885	3,380	
2-3	"		0 99412	\times 0 6978	2,982	
3-4	"		0 99110	\times 0 6578	2,802	
4-5	"		0 98827	\times 0 6306	2,664	16,128
5-10	43×5	\times	0 97959	\times 0 5748	12,105	
10-15	"		0 96522	\times 0 5445	11,299	39,532
15-20	"		0 94330	\times 0 5097	10,591	
20-25	"		0 93440	\times 0 4771	9,584	
25-30	"		0 92073	\times 0 4294	8,502	
30-35	"		0 90725	\times 0 3758	7,311	
35-40	"		0 89397	\times 0 3195	6,140	
40-45	"		0 88090	\times 0 2716	5,143	
45-50	"		0 86800	\times 0 2241	4,182	90,791
50-55	"		0 85530	\times 0 1794	3,111	
55-60	"		0 84270	\times 0 1328	2,409	
60-65	"		0 83040	\times 0 0943	1,683	
65-70	"		0 81820	\times 0 0613	1,078	
70-75	"		0 80630	\times 0 0344	596	
75-80	"		0 79440	\times 0 0137	234	
80-85	"		0 78230	\times 0 0044	74	
85-90	"		0 77130	\times 0 0010	18	
90-95	"		0 76000	\times 0 0002	2	100,000

TABLE IX

Probability of surviving

	l_x	d_x	q_x
0—1	10,000	2,115	0 2415
1—2	7,585	607	0 0798
2—3	6,978	400	0 0573
3—4	6,578	272	0 0414
4—5	6,306	198	0 0315
5—6	6,108	148	0 0243
6—7	5,960	113	0 0190
7—8	5,847	99	0 0170
8—9	5,718	86	0 0150
9—10	5,662	89	0 0140
10—11	5,583	72	0 0130
11—12	5,511	66	0 0120
12—13	5,445	68	0 0125
13—14	5,377	70	0 0130
14—15	5,307	70	0 0132
15—16	5,237	70	0 0134
16—17	5,167	70	0 0136
17—18	5,097	70	0 0138
18—19	5,027	70	0 0140
19—20	4,957	186	0 0142
20—25	4,771	177	0 0200
25—30	4,294	536	0 0250
30—35	3,758	563	0 0290
35—40	3,195	479	0 0310
40—45	2,716	475	0 0350
45—50	2,241	447	0 0420
50—55	1,794	436	0 0500
55—60	1,328	385	0 0680
60—65	943	330	0 0700
65—70	613	269	0 0880
70—75	344	207	0 1200
75—80	137	93	0 1800
80—85	44	34	0 2700
85—90	10	8	0 3800
90—95	2		

A FURTHER STUDY ON THE VITAL CAPACITY OF SOUTH INDIANS

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IN a former publication in this *Journal* (Krishnan and Vareed, 1932) we analysed the vital capacities of 103 male medical students according to age, height, weight, body surface, etc , and discussed the correlation existing between the vital capacity and the various measurements. The Lownes spirometer (dry type) was then used for the estimation of the vital capacity, and the average was found to be 2.93 litres which is 69.6 per cent of the average found by Jackson and Lees (1928) for American men of the same height as the Indian mean height, but 72.9 per cent of the average of the Americans of the same mean body surface area. The opinion was then put forward that the lower standards obtained, compared with those of the Americans, are due not to racial differences but to the influence of the hot climate resulting in low metabolism and less tendency for exercise.

Since that publication, a paper has appeared on 'Standards for predicting the normal vital capacity in South Indian women from height, weight and surface area' (Mason, 1932). Using the Collins water spirometer, Mason found the average for South Indian women to be 2.15 litres which, she said, is 76 per cent of the average for American women of the same height as the Indian mean height. But on calculation it is found that the average 2.15 litres is only 70.5 per cent of the American average for the Indian mean height obtained in her series. In her paper Mason remarked in a footnote that it is unlikely that the average for South Indian men is as low as the figure obtained in our series and that, if out of 103 South Indians 9 Anglo-Indians are excluded, the average would be 2.86 litres which is 67 per cent of the average for American men of the same height as the Indian mean height. If the body surface area is taken into consideration, the average 2.86 litres obtained

for men is 71.2 per cent of the American average for men and 2.15 litres obtained by Mason for women is 76 per cent of the American average for women. The reason for including the Anglo-Indians in our first series, we shall deal with later in this paper.

In order to ascertain if our figures were really low, the vital capacities of a second series of 260 medical students (ranging from 17 to 26 years), including 39 Anglo-Indians and 23 women, were estimated with the Sanborn water spirometer which has as good a reputation as the Collins spirometer used by Mason. The usual precautions were taken to ensure accuracy of record and in each case the highest reading in three or four trials was taken as the vital capacity. Other physical measurements of the body such as standing height, weight, body surface area (from Aub-DuBois chart), mean chest girth, and chest expansion, were also recorded.

TABLE I

Average vital capacity of 198 men, excluding Anglo-Indians, according to standing height

Height in cm	Number of subjects examined	Average vital capacity in litres
150	2	2.67
155	21	2.73
160	32	2.75
165	70	3.03
170	55	3.24
175	16	3.48
180	1	3.68
185	<i>nil</i>	.
190	1	3.20
AVERAGES 165	.	3.05

Vital capacity per cm. of height is 18.5 c.c.

TABLE II

Average vital capacity of 198 men, excluding Anglo-Indians, according to weight

Weight in kilos	Number of subjects examined	Average vital capacity in litres
40	7	2 63
45	31	2 77
50	59	3 04
55	45	3 11
60	32	3 19
65	15	3 33
70	2	3 66
75	7	3 20
AVERAGES 53 9		3 05

TABLE III

Average vital capacity of 198 men, excluding Anglo-Indians, according to body surface

Body surface in sq m	Number of subjects examined	Average vital capacity in litres
1 30	2	2 70
1 40	25	2 64
1 50	47	2 94
1 60	64	3 08
1 70	41	3 19
1 80	15	3 47
1 90	4	3 45
AVERAGES 1 59		3 05

TABLE IV
*Average vital capacity of 198 men, excluding Anglo-Indians,
 according to age*

Age	Number of subjects examined	Average vital capacity in litres	Average vital capacity per sq m in litres
17	2	2 22	1 80
18	9	2 93	1 91
19	38	3 03	1 91
20	43	3 03	1 94
21	44	3 09	1 91
22	30	3 07	1 94
23	21	3 23	2 01
24	9	3 05	1 97
25	1	2 80	1 81
26	1	2 51	1 72

TABLE V
*Average vital capacity of men, excluding Anglo-
 Indians, according to mean chest measurement*

Mean chest girth in cm	Number of subjects examined	Average vital capacity in litres
72	2	2 48
74	2	2 85
76	10	2 98
78	6	2 87
80	8	3 40
82	4	3 11
84	8	3 35
86	1	2 90
88	4	3 27
90	1	2 50
92	1	3 64

TABLE VI

Average vital capacity of men, excluding Anglo-Indians, according to chest expansion

Chest expansion in cm	Number of subjects examined	Average vital capacity in litres
3	1	2.40
4	4	2.90
5	8	3.14
6	12	3.15
7	6	2.89
8	7	3.48
9	5	3.36

Tables I to VI represent the average vital capacities of 198 South Indian men, excluding Anglo-Indians, according to height, weight, body surface, age, chest circumference (mean of measurement on deep inspiration and that on deep expiration) and chest expansion. The average vital capacity of men in this series excluding Anglo-Indians is found to be 3.05 litres, 1.93 litres per sq m of surface area and 18.5 c.c. per cm of height. In general these figures are about 5 per cent higher than the figures obtained in our first series. As Myers observed, individual variation offers one of the greatest limitations of vital capacity test. The type of the spirometer also seems to be responsible for slight variations in the figures obtained. In view of these variations, it is generally recognized that a 10 per cent margin should be allowed in vital capacity measurements, and our figures in the first series were only 5 per cent lower than the averages obtained in the present series. Turner (1927) in her first series for American women obtained an average of 2.99 litres and in her second series (1930), using the Collins spirometer, obtained 3.28 litres, and for American men Jackson (1927) found 4.383 litres as the general standard average, and he and Lees (1928) using the Sanborn water spirometer found 4.406 litres as the average for 100 male students. The object of measuring the vital capacity in different groups of people is only to determine the standard for such groups, and as long as the figures obtained indicate a certain standard, no serious note need be taken of slight variations within 10 per cent.

In the present series the average obtained for 198 men excluding Anglo-Indians is 3.05 litres which is 75.4 per cent of the average of American men of the same surface area as the Indian mean surface area as compared with 71.2 per cent obtained in the first series.

It is generally recognized that vital capacity is closely related to the surface area. Peabody and Wentworth (1917) found a close correlation between the two, and Dreyer (1919) said that the vital capacity is a simple function of the body surface area and not of height. West (1920) also advised the

vital capacity was nearly equal to the Western standard. It will be seen from the above analysis that the Anglo-Indian students are in no way different from other Indians, who had good physique and chest expansion as a result of physical training and there is sufficient justification for including them in the series as South Indians and for striking a general average. If the Anglo-Indians are also included in this series the average vital capacity for a total number of 237 South Indian men would be 3.17 litres (1.99 litres per square metre) which is 77.4 per cent of the American average for the same surface area as the Indian mean surface area.

In the present series, the vital capacities of 23 women students of 18 to 23 years were also measured and Table IX represents their average vital capacity according to standing height. With a mean height of 154.5 cm and a mean surface area of 1.42 sq m then average vital capacity is found to be 2.20 litres, 1.55 litres per sq m of surface area, and 14.2 c.c. per cm of height. This general average (2.20 litres) is 74.6 per cent of the average for American women of the same mean body surface area.

TABLE IX

*Average vital capacity of 23 women students
according to height*

Height in cm	Number of subjects examined	Average vital capacity in litres
145	2	2.04
150	9	2.12
155	9	2.32
160	3	2.20
AVERAGES 154.5		2.20

Average vital capacity per cm of height is 14.2 c.c.

Mean body surface area is 1.42 sq m

Average vital capacity per sq m is 1.55 litres

Mason (1932) refers in her paper to a variety of races in South India and the variations she found in the vital capacities of the Tamils, Telugus and Malayalis. In our series for men, the data obtained for Tamils, Telugus, Malayalis and Canarese are shown in Table X, from which it will be seen that the average

vital capacities for the different groups are 3.01, 3.05, 3.07, and 3.03 litres respectively, which are very near or the same as the general average (3.05 litres) when reduced to the same mean body surface

TABLE X

Data concerning the four groups of South Indians speaking different languages

Language spoken	Number of subjects examined	Mean height in cm	Weight in kilos	Body surface in sq m	Vital capacity in litres	Vital capacity per sq m in litres
Tamils	69	166	53.3	1.62	3.01	1.90
Telugus	15	165	52.9	1.57	3.05	1.94
Malayalis	70	164	53.6	1.57	3.07	1.95
Canarese	24	166	54.9	1.60	3.03	1.95

South Indians speaking different Dravidian languages are all of one race, and any slight variation found in different groups of persons can only be the result of the varying mode of life and habits. The variations observed by Mason in the different groups of women and those observed in our groups of men are too small to warrant any serious consideration of racial differences. The small increase in the vital capacity of the Malayalis could be accounted for by the influence of a more equitable climate on the West Coast where they come from, and their habits of staying more in the open. Dealing with women especially, it has to be borne in mind that besides the influence of climate and habits, variation in metabolism, resulting from endocrine activity from the time of puberty onwards, is bound to contribute to small changes in their physiological measurements such as vital capacity.

Racial differences, if any, should be more anatomical than physiological. If anatomically two races are found to be the same, any physiological variation can only be due to adaptation to the surroundings and habits acquired.

SUMMARY

1. A further study was made on the vital capacities of 260 South Indian medical students including 39 Anglo-Indians and 23 women, using the Sanborn water spirometer.

2. The average vital capacity of 198 men excluding Anglo-Indians is found to be 3.05 litres, 1.93 litres per sq m of body surface and 18.5 c.c. per cm of standing height. In general the figures are 5 per cent higher than the figures obtained in our first series. The average 3.05 litres is 75.4 per cent of the average for American men of the same surface area as the Indian mean surface area.

3 The average for 39 Anglo-Indians is found to be 3.78 litres which is 89.0 per cent of the average for Americans. The high average in these cases is considered to be due not to any racial difference but to the habit of indulging in constant exercise. The average for 35 other Indians known to indulge in constant exercise is found to be practically the same as that of the Anglo-Indians. The general average for South Indian men including the Anglo-Indians works out to be 3.17 litres (1.99 litres per sq m) which is 77.4 per cent of the American average.

4 The average vital capacity of 23 women students is found to be 2.20 litres which is 74.6 per cent of the average for American women of the same surface area as the Indian mean body surface area.

5 The importance of the correlation of the vital capacity with the surface area and the applicability of the DuBois formula to Indians is discussed.

6 It is pointed out that the South Indians, speaking the Dravidian languages, are all of one race, and any small variations in the vital capacities of the Tamils, the Telugus, the Malayalis and the Canarese are only due to the varying modes of life and habits.

We wish to express our indebtedness to Rao Bahadur Dr M. Kesava Pai, M.D., O.B.E., Superintendent of the Government Tuberculosis Hospital, Madras, for kindly lending us the Sanborn water spirometer for measuring the vital capacities.

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AN INVESTIGATION INTO THE CLINICAL VALUES OF THE LÆVULOSE AND THE GALACTOSE TOLERANCE TESTS FOR HEPATIC FUNCTION

BY

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THE lævulose and the galactose tolerance tests for determining hepatic efficiency are not new. Ever since Claude Bernard discovered the glycogenic function of the liver in 1857, attempts have been made to test this function in determining hepatic efficiency. Strauss (1901) introduced lævulose for testing hepatic function on the basis of the experimental work of Sachs (1899) who found that the metabolism of lævulose was disturbed to a great extent in de-hepatized frogs. Strauss (1901) method consisted in examining the urine for lævulose after the ingestion of 100 grammes of the sugar. He stated that the test was positive in 90 per cent of cases of hepatic disorders whilst only 10 per cent of the healthy controls showed lævulosuria. Divergent views were expressed about the value of this test, and in its original form the test is unreliable as it involves a renal factor (renal threshold for lævulose lies between 0.115 per cent and 0.13 per cent—Tallerman, 1923). The test was modified by Schirokauer (1913) by estimating the blood-sugar after the administration of lævulose. He found that in healthy controls the rise of blood-sugar above the fasting level, one hour after the ingestion of 100 grammes of lævulose, was very little, while in cases in which the liver was damaged the blood-sugar rose even up to 0.19 per cent. The work of Maclean and de Wesselow (1920, 1921) on the estimation of sugar tolerance also showed that the rise of blood-sugar in normal individuals after the ingestion of lævulose was not at all marked. Spence and Brett (1921) also pointed out that the lævulose tolerance test is valuable in determining hepatic efficiency.

Richard Bauer (1906) of Vienna introduced galactose for testing the glycogenic function of the liver. He showed that in cases with damage or destruction of the hepatic parenchyma the administration of 40 grammes of galactose by mouth resulted in galactosuria. In all the cases of portal cirrhosis, catarrhal and toxic

jaundice, Bauer (1908, 1912) obtained positive results, while he found that the test was negative in cases of obstructive jaundice due to gall-stones, pressure of tumours, etc. He pointed out that cases of catarrhal and toxic jaundice can thus be differentiated from cases of obstructive and hæmolytic jaundice by means of this test. The importance of this test in the differential diagnosis of jaundice has also been pointed out by Shay, Schloss, and Rodis (1931), who obtained similar results. Davies (1927) expressed similar views by studying the blood-sugar curves after the ingestion of galactose. Bauer placed considerable reliance on the urinary findings alone and says, 'galactosuria is often the only positive sign of abnormal liver function'. The considerations underlying the use of galactose in the tests of hepatic function are discussed in detail by Shay, Schloss, and Bell (1931).

Both these tests depending on the carbohydrate metabolism of the liver have been the subject of much discussion in the recent literature. There is also much difference of opinion about the comparative value of both these tests. Elmer and Scheps (1930) compared the blood-sugar curves after the administration of lævulose and galactose in the same individuals with hepatic disorders and found that in most of the cases they were identical, but a few cases were encountered in which the lævulose test, in the presence of a positive galactose test, was slightly positive or completely negative, and hence they concluded that the galactose test is much more valuable than the lævulose test. They (Elmer and Scheps, 1928, 1930) found that the galactose tolerance test as described by Bauer (estimation of galactose in urine) is unreliable, as they found cases in which the rise of blood-sugar exceeded 30 mg per cent after the administration of galactose and yet there was little or no sugar at all in the urine. Davies (1927), Kahler and Machold (1922) also pointed out that in the galactose tolerance test it is misleading to depend upon the urinary findings alone to determine the hepatic efficiency and considered the blood-sugar values to be more important.

Tallerman (1923), Covell (1923), Finkelstein and Dannenberg (1925), King (1927), and Hurst (1931) among others considered the lævulose tolerance test to be of considerable value as an index of the functional capacity of the liver. De Wesselow (1924) pointed out that in bad cases of hepatic damage, the blood-sugar curve obtained after the ingestion of lævulose may closely resemble that of a mild diabetic after the administration of a corresponding dose of glucose. On the other hand, Rowntree, Marshall, and Chesney (1914) and Greene, McVicar, Snell, and Rowntree (1927) pointed out the unsatisfactory character of the fructose (lævulose) tolerance test in cirrhosis of the liver. The unsatisfactory nature of this test for hepatic function has also been pointed out by Greene, Snell, and Walters (1925). The observations of Hughes and Malik (1930) on a number of hospital patients in Lahore showed that the lævulose tolerance test was positive in most of them, though there was no clinical evidence of hepatic disease. They suggested that the diminished tolerance for lævulose in these patients was the result of defective formation and storage of glycogen in the liver, probably due to previous attacks of malaria, from which they had all suffered.

To determine the clinical values of the lævulose and galactose tolerance tests for hepatic efficiency, especially in cases of cirrhosis of the liver, a number of patients was investigated in the medical wards of the King George Hospital, Vizagapatam, and the results obtained in them are detailed in this paper.

TECHNIQUE

(A) *Lævulose tolerance test*

The test is always done in the morning before the patient takes his breakfast, and is preceded by a twelve-hour fast. A sample of blood is drawn from the vein to estimate the initial fasting blood-sugar. Lævulose dissolved in water is then given by mouth, according to the weight of the patient as recommended by Spence and Brett (1921). Four specimens of blood are then collected at half-hourly intervals and the blood-sugar is estimated by the method of Folin and Wu (1920). The lævulose supplied by Hopkin and Williams, Ltd, London, was used in this investigation.

King (1927), Elmer and Scheps (1930) showed that in normal persons the maximum rise of blood-sugar above the fasting level never exceeded 20 mg per cent. According to Tallerman (1923), a maximum rise of 30 mg of blood-sugar above the initial fasting level, or a blood-sugar reading exceeding 0.135 g per cent, indicated a definite derangement of hepatic function. Adopting these standards the lævulose tolerance test is considered negative if the maximum rise of blood-sugar does not exceed 20 mg per cent and positive if it exceeds 30 mg per cent, if the maximum rise of blood-sugar is in between these two readings (i.e., between 20 and 30 mg per cent), the test is considered as doubtful.

(B) *Galactose tolerance test*

The technique of this test is described in detail by Shay, Schloss, and Rodis (1931). The patient is prepared in the same way as in the lævulose tolerance test. On the morning of the test the patient is asked to pass urine and it is examined qualitatively for sugar. Forty grammes of pure galactose dissolved in about 15 ounces of water is then given by mouth, irrespective of the weight of the patient. The patient is instructed to pass urine every hour or as closely to the hourly periods as possible, during the five hours following the administration of galactose. During this period no food is allowed to the patient and, if desired, water is allowed to drink, as it was found not to interfere with the result. The total quantity of urine passed during the five-hour period is measured and is examined qualitatively for sugar, if positive, the total amount of sugar in the urine is estimated by Benedict's method (as described by Cole, 1926). The total quantity of galactose excreted in the urine is then calculated after the necessary correction is made for the copper reduction by galactose. In the case of diabetic persons the galactose excreted in the urine is determined after the dextrose present in it is destroyed by rapid fermentation with a yeast suspension.

There is difference of opinion about the output of galactose which should be accepted as normal after the administration of 40 g of the sugar. 'Bauer tabulated his urinary findings as follows: Normal, 0-1 g, high normal, 1-2 g, positive, 2-3 g, high positive, 3-12 g. The greatest loss that has been observed in this series is 6.5 g (Davies, 1927)'. Davies (*loc cit*) concluded that any amount of over 2 g indicates a definite hepatic deficiency. Shay and Schloss (1931) considered an output of more than 3.0 g of galactose in the five-hour period following the administration of 40 g of galactose, as indicative of impaired hepatic function.

RESULTS

In the present study only those patients in whom the clinical picture was typical and the diagnosis definite were selected for investigation. The clinical diagnosis was verified at operation in one case (No 8) and at autopsy in five cases (Nos 107, 138, 151, 136 and 127). In most of the cases the McNee and Keefer's (1925) modification of the van den Bergh's reaction (1918) and the 'Rose Bengal' test for liver function were also performed. The interpretation of the results of the 'Rose Bengal' test is given in a separate paper (Radhakrishna Rao, 1933). It may be pointed out here that the 'Rose Bengal' test gives a fairly good idea of the hepatic efficiency. Cases complicated by endocrine disturbances are not included in this series except one case (No 81) of diabetes mellitus.

The results of the lævulose tolerance test performed on 45 patients are tabulated in Table I.

Variable results were obtained in cases of cirrhosis of the liver. Thus, out of the 28 cases, the lævulose tolerance test was positive in 21, doubtful in 4 and negative in 3 cases (Nos 1, 8 and 19). In three cases of catarrhal jaundice, the test was doubtful in two cases and positive in one case (No 81) in which the fasting blood-sugar was very high and sugar was present in the urine. The test was negative in one case of toxic jaundice complicating pregnancy, it was positive in one case of jaundice associated with avitaminosis and in both the cases of jaundice due to malignant disease of the liver. Negative results were obtained in three cases of anæmia due to ankylostomiasis, while in one case (No 83) in which there was marked retention of the dye 'Rose Bengal', the test was doubtful. In two cases of ascites associated with congestive heart failure due to bad myocarditis, the test was negative in one (case No 23) and doubtful in another (case No 80). Cases of ascites due to peritonitis gave varied results, in one case (No 62) of simple chronic peritonitis the test was doubtful, while in another case (No 127) of malignant peritonitis in which the diagnosis was verified post-mortem, the test was positive, the test was negative in one case (No 150) of tubercular peritonitis. In case No 111 in which there was marked retention of 'Rose Bengal', the lævulose tolerance test was negative.

Thus, out of the 37 cases in which there was definite evidence of hepatic dysfunction, the test was positive in 25, doubtful in 7 and negative in 5 cases. While, in the 8 cases in which there was no evidence of hepatic disease, the test was positive in 1, doubtful in 2 and negative in 5 cases.

In most of the positive cases, the maximum rise of blood-sugar above the fasting level was found at the end of one to one and half hours and the blood-sugar curve showed no tendency to return to the fasting level at the end of two hours.

Table II shows the results of the galactose tolerance test in some typical cases in most of which the 'Rose Bengal' and the lævulose tolerance tests were also performed for comparison. The galactose tolerance test was positive in one case (No 157) of jaundice in which there was evidence of diffuse damage of the hepatic parenchyma due to 'Neo-Salvarsan', administered for the treatment of syphilis, while, in another case (No 99) of toxic jaundice complicating pregnancy, the test was negative, though there was moderate retention of 'Rose Bengal'. Out of the 9 cases with definite evidence of chronic liver cell damage, the test was positive only in 3 cases (taking 2.0 g. as the maximum permissible limit of the output of galactose

in normal persons), 3 cases (Nos 119, 79 and 72) showed a normal 'Rose Bengal' test indicating that there was still sufficient amount of actively functioning liver-tissue. In case No 107, the diagnosis was verified post-mortem, in this case, though the 'Rose Bengal' test showed marked retention and the lævulose tolerance test was positive, the galactose tolerance test gave a negative result. Out of the 7 cases of cirrhosis of the liver, the galactose tolerance test was positive in 3 and negative in 4 cases.

In this paper no attempt is made to study the value of the galactose tolerance test in the differential diagnosis of jaundice, how far the test is valuable in determining hepatic efficiency is only considered here. The blood-sugar curves after the ingestion of galactose are not studied in this paper.

COMMENT

Both the lævulose and the galactose tolerance tests depend on the functional activity of the liver in relation to carbohydrate metabolism. Several factors are concerned in the regulation of the carbohydrate metabolism of the body, besides the liver, the muscles, the islet tissue of the pancreas and the anterior lobe of the pituitary play an important part. But, as far as the metabolism of lævulose and galactose are concerned, they are solely metabolized in the normal liver and the other factors regulating the carbohydrate metabolism do not apparently come into play. Hence, after the ingestion of a given amount of lævulose or galactose in a normal person, there is little appreciable rise in the blood-sugar level. When the liver is diseased, the liver cells are not able to deal efficiently with these sugars and convert them into glycogen, and hence they escape into the general circulation unchanged, resulting in a rise in the blood-sugar content. As there is no renal threshold for galactose (Shay, Schloss, and Bell, 1931, Rowe and Chandler, 1924), any slight rise in the blood-sugar content due to galactose, results in its excretion in the urine.

Though the principles underlying the use of these tests are so simple and their results so promising, the clinical application of the tests in cases of diseases of the liver, is productive of widely variant results. These divergent results are due to the fact that the liver has a great reserve power and a remarkable capacity to regenerate, especially in chronic liver-cell damage. Mann and Bollman (1926) showed that after removing 70 per cent of the normal liver of a dog, the remaining liver-tissue increased and returned approximately to its pre-operative level in a few weeks time. Thus, in chronic diseases of the liver (e.g., cirrhosis), a sufficient amount of actively functioning liver-tissue is still present to maintain the carbohydrate metabolism. The functional aspects of the regenerated hepatic tissue have recently been reported by Althausen (1931). He showed that in toxic cirrhosis of the liver, where there is marked regeneration of the liver-tissue, the 'carbohydrate metabolism-regulating function' of the liver remains normal, though there is marked impairment in the excretion of 'Rose Bengal'. Thus, tests depending on the carbohydrate function of the liver become positive only when there is diffuse destruction of the hepatic parenchyma, either acute or chronic, and when there is failure of regeneration in the chronic cases.

The experimental evidence in support of the lævulose tolerance test for hepatic efficiency is also conflicting. Bodansky (1923) obtained positive results in dogs in

which the liver was experimentally damaged. On the other hand Mann and Bollman (1926) showed in their experimental study on liver function tests that in dogs in which the hepatic tissue was permanently reduced the lævulose tolerance test failed to bring out any marked deviation from the normal.

The results given in Table I show that the lævulose tolerance test gave positive results in a greater number of cases with liver disease than in those without evidence of hepatic disorder. As the test gave negative results in some of the cases (e.g., Nos. 1, 8, 19 and 99) with definite evidence of liver disease, it cannot be considered to be of diagnostic value in any individual case. Moreover, while a positive test is more in favour of hepatic dysfunction, a negative test cannot exclude liver disease, especially in chronic cases. Clinical evidence of disease of the liver is generally present by the time the lævulose tolerance test gives positive results and the test then is more confirmatory rather than an aid in the diagnosis. Tests of this type depending on the partial function of the liver in the regulation of the blood-sugar level 'do not permit of an exact quantitative estimate of the degree of impairment of liver function' (de Wesselow, 1924). The liver has varied functions and the failure of its function in carbohydrate metabolism does not necessarily indicate that its other functions are also impaired. As pointed out by Althausen (1931), the carbohydrate metabolism of the liver remains normal in cases of toxic cirrhosis though the excretion of the dye is markedly impaired. Kahler and Machold (1922) investigating the value of galactose for liver function, concluded that hyperglycæmia and galactosuria denote only the derangement of the hepatic control over carbohydrate metabolism and their value in the estimation of hepatic efficiency remains to be established.

As pointed out by King (1927), the height and duration of the blood-sugar curve after the ingestion of lævulose is influenced, apart from the functional capacity of the liver, by the rate of absorption of lævulose from the intestines, the condition of the pancreas and the rate of elimination of the sugar by the kidneys. Though the pancreas is not directly concerned in the metabolism of lævulose, in cases of diabetes where pathological changes have taken place in the islet tissue of the pancreas the results of the lævulose tolerance test may be considerably influenced by the pancreatic deficiency, hence, the test should be considered as abnormal only in the absence of pancreatic disease.

It has not been possible to estimate lævulose as such in the blood. The total blood-sugar estimated at the half-hourly periods after the administration of lævulose, includes the glucose content of the blood and the lævulose which has escaped into the general circulation. Thus, it will be noted that the results of this test will be vitiated unless the lævulose used is pure and is free from any contamination with glucose.

Kahler and Machold (1922), Davies (1927), Rolleston and McNee (1929), Elmer and Scheps (1930), Dodds (1931) and Bode (1931) among others considered the blood-sugar curves in the galactose tolerance test to be of value in the diagnosis of hepatic disorders. The unsatisfactory nature of the urinary findings in the galactose tolerance test has been pointed out (as mentioned before) by Kahler and Machold (1922), Davies (1927) and Elmer and Scheps (1930). 'Alone, this observation on the urine is open to many errors, as the threshold varies enormously in normal people, while in nephritic cases the kidney may entirely fail to excrete it. In diabetes the test is, of course, valueless. Again, if much ascites is present, it has been shown

that the absorption of the sugar in the alimentary tract may be delayed, and the sugar still excreted even for five to six hours following the ingestion (Davies, 1927)'

Though the results obtained in this study are not sufficient to draw adequate conclusions, it may be stated that the total amount of galactose excreted in the urine is too variable to consider it as a guide to hepatic efficiency

The galactose tolerance test of Bauer has its own advantages. It is a simple test which is easily performed without much discomfort to the patient and there are no contra-indications except diabetes mellitus. In cases in which it is not possible to obtain blood for the estimation of the blood-sugar after the administration of galactose this test may be performed to gauge roughly the efficiency of the liver.

SUMMARY AND CONCLUSIONS

(1) To determine the clinical values of the *lævulose* and the galactose tolerance tests for estimating hepatic efficiency, a number of patients was investigated in the medical wards of the King George Hospital, Vizagapatam, and the results obtained are discussed.

(2) The *lævulose* tolerance test gave positive results in a greater number of cases with liver disease than in those without evidence of hepatic disorder. In some cases with definite evidence of liver disease the test gave negative results, and hence it is concluded that the test cannot be considered to be of diagnostic importance in any individual case.

(3) The variable results obtained by the *lævulose* tolerance test in cases of cirrhosis of the liver are considered to be due to the regeneration of the hepatic tissue. It is pointed out that a negative test in these cases cannot exclude liver disease, as it only indicates that a sufficient amount of actively functioning liver-tissue is still left to maintain the carbohydrate metabolism. The test becomes positive only when there is diffuse destruction of the hepatic parenchyma together with failure of regeneration.

(4) Though the galactose tolerance test of Bauer (1906) has its own advantages, it is misleading to depend on the urinary findings alone and the total amount of galactose excreted in the urine is too variable to consider it as a guide to hepatic efficiency.

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TABLE I

Showing the results of the lavulose tolerance test

Serial number	Case number	LÆVULOSE TOLERANCE TEST (BLOOD SUGAR IN MG PER CENT)					Dye retention (Rose Bengal) at the end of 8th minute (per cent)	VAN DEN BERGH'S REACTION		Diagnosis	Basis of diagnosis	REMARKS
		Initial	½ hour	1 hour	1½ hours	2 hours		Direct	Indirect			
1	81	196.0	211.6	227.2	215.0	195.2	31.2	Immediate	Positive	Catarhal jaundice	Clinical	Sugar present in the urine
2	129	73.5	100.0	98.0	98.0	97.6	26.5	"	"	"	"	"
3	122	88.9	114.9	108.1	93.0	85.5	26.0	"	"	"	"	"
4	99	73.5	74.1	74.3	85.8	74.1	12.3	"	5.05 units	Toxæmia of pregnancy—jaundice	"	"
5	100	108.1	137.0	153.8	135.1	130.7	45.7	"	9.375 "	Avitaminosis and jaundice	"	"
6	92	145.6	161.2	201.2	263.2	256.4	117.6	Biphasic	Positive	Primary malignant disease liver—jaundice	"	"
7	119	104.2	133.3	137.0	144.9	137.9	40.7	Immediate	16.5 units	Secondary malignant deposits liver—jaundice	"	Primary growth in the retained testicle on the right side
8	2	78.4	96.6	119.0	126.6	119.7	48.2	Delayed	Positive	Portal cirrhosis liver—ascites	"	"
9	7	89.7	107.5	140.8	150.4	139.9	60.7	"	"	"	"	"

TABLE I—*contd*

Serial number	Case number	LÆVULOSE TOLERANCE TEST (BLOOD SUGAR IN MG PER CENT)					Maximum rise of blood sugar (mg per cent)	Dye retention (Rose Bengal) at the end of 8th minute (per cent)	VAN DEN BERGH'S REACTION		Diagnosis	Basis of diagnosis	REMARKS
		Initial	½ hour	1 hour	1½ hours	2 hours			Direct	Indirect			
10	8	83.0	76.6	83.0	99.0	101.5	18.5		Delayed	Positive	Portal cirrhosis liver—ascites	Operation	Omentopexy done
11	26	68.9	79.2	102.4	116.5	106.4	47.6		Immediate	8.065 units	"	Clinical	
12	47	79.7	110.5	121.2	113.6	101.5	41.5		Negative	Negative	"	"	
13	51	78.5	96.4	97.5	116.5	99.6	38.0		Delayed	Positive	"	"	
14	52	126.6	153.8	156.2	117.6	125.0	29.6		Negative	"	"	"	
15	79	88.9	97.1	102.6	111.7	107.5	22.8	55.8	Delayed	"	"	"	
16	97	79.4	103.6	117.6	87.3	113.6	38.2	92.6	"	"	"	"	
17	107	58.4	59.5	94.3	95.7	102.0	43.6	98.6	"	3.3 units	"	Necropsy	Liver multilobular cirrhosis with fatty degeneration (microscopic examination)
18	131	108.1	129.0	134.2	112.4	93.9	26.1	72.1			"	Clinical	

	19	138	85.8	116.3	109.5	153.2	109.3	83.7	88.2	Immediate	Positive		Necropsy	Multilobular cirrhosis of the liver (micro scopic examina- tion)
20	151	95.7	134.2	135.8	137.9	137.9	137.9	42.2	40.4	Biphasic	5.88 units	"	"	Multilobular cirrhosis of the liver (micro scopic examina- tion)
21	135	59.7	77.8	85.5	84.9	82.0	82.0	25.8	98.0	"	3.125 "	"	Clinical	
22	121	68.7	77.5	77.6	86.9	100.5	100.5	31.8	98.4	Delayed	Positive	"	"	
23	125	87.7	112.4	137.0	137.9	115.6	115.6	50.2	76.5	Biphasic	6.755 units	"	"	
24	142	83.2	133.3	136.0	108.1	118.3	118.3	49.8	79.8	Immediate	8.67 "	Biliary cirrhosis (non obstructive)— no ascites	"	
25	1	80.2	105.3	95.2	95.2	86.0	86.0	19.1		Delayed	Positive	Syphilitic cirrhosis liver—ascites	"	
26	19	88.9	85.1	103.6	89.7	90.9	90.9	14.7		"	"	"	"	
27	24	70.2	133.3	125.0	101.0	82.6	82.6	63.1		Biphasic	"	"	"	
28	94	103.6	130.9	137.0	232.6	114.9	114.9	129.0	66.6	Negative	"	"	"	
29	72	73.3	79.4	84.0	74.1	114.9	114.9	41.6	57.5	"	Negative	Syphilitic cirrhosis liver and polysero- sitis	"	

TABLE I—concl'd

Serial number.	Case number	LÆVULOSE TOLERANCE TEST (BLOOD-SUGAR IN MG PER CENT)					Dye retention (Rose Bengal) at the end of 8th minute (per cent)	VAN DEN BERGH'S REACTION		Diagnosis	Basis of diagnosis	REMARKS
		Initial	½ hour	1 hour	1½ hours	2 hours		Direct	Indirect			
30	134	125.0	139.9	212.8	200.0	140.8	88.6	Delayed	1.5 units	Syphilitic cirrhosis liver—ascites	Clinical	
31	123	83.3	100.0	110.5	105.8	105.8	98.5	Immediate	7.935 "	Syphilitic cirrhosis liver—no ascites	"	
32	153	62.7	77.5	106.9	95.2	83.3	71.4	"	6.665 "	"	"	
33	84	62.3	79.7	90.9	100.0	92.6	95.0	"	6.87 "	Biliary cirrhosis (non obstructive)—ascites	"	
34	44	56.3	61.9	89.3	95.2	86.2		"	18.1 "	Biliary cirrhosis (non obstructive)—no ascites	"	
35	136	42.3	70.1	81.1	61.5	72.1	61.2	Negative	Positive	Capsular cirrhosis liver—polyserositis	Necropsy	Perihepatitis and early capsular cirrhosis of the liver (Microscopic examination)
36	128	96.1	106.4	107.8	95.9	95.4	66.7	Delayed, faint	Positive, faint	Ankylostomiasis—anaemia—no ascites	Clinical	

37	46	69.2	64.5	67.1	66.6	66.6	No rise	Negative	Negative	Ankylostomiasis— anemia—ascites	"	
38	53	89.7	101.0	108.7	92.2	102.6	19.0	Delayed, faint	Positive, faint	"	"	
39	83	111.7	111.1	135.1	119.0	118.3	23.4	Delayed	Positive	"	"	Fatty degeneration liver?
40	62	78.7	81.3	81.4	102.6	94.3	23.9	Negative	Positive, faint	Chronic peritonitis— ascites	"	
41	80	94.3	109.9	116.3	107.5		22.0	"	Negative	Myocarditis—ascites	"	
42	23	80.0	88.9	90.9	94.3	77.5	14.3	"	"	Syphilitic myocarditis—ascites	"	
43	127	82.0	121.2	102.0	93.9	93.0	39.2	Delayed, faint	Positive, faint	Malignant peritonitis—ascites	Necropsy	Liver (microscopically) slight peritonitis—otherwise normal Malignant peritonitis secondary to fibrosarcoma of the mesentery
44	150	79.7	86.2	87.3	95.2	98.5	18.8	Negative	Negative	Tubercular peritonitis—ascites—bilateral pleural effusion	Clinical	
45	111	68.3	73.0	76.2	73.0	69.0	7.9	Biphasic	2.5 units	Enlarged liver and spleen—chronic malaria—no ascites	"	

TABLE II

Showing the results of the galactose tolerance test in some typical cases

Serial number	Case number	Total quantity of galactose excreted (g.)	Lævulose tolerance test—maximum rise blood sugar (mg per cent)	Dye retention (Rose Bengal) at the end of 8th minute (per cent)	VAN DEN BERGH'S REACTION		Diagnosis	Basis of diagnosis	REMARKS
					Direct	Indirect			
1	83	0.214	23.4	92.6	Delayed	Positive	Ankylostomiasis—anaemia and ascites	Clinical	(Fatty degeneration liver?) No jaundice
2	62	0.38	23.9	59.5	Negative	Positive, faint	Chronic peritonitis—ascites	"	No jaundice
3	119	0.51	40.7	57.1	Immediate	16.5 units	Secondary malignant deposits—liver	"	Jaundice present
4	99	0.05	12.3	78.6	"	5.5 "	Toxaemia of pregnancy—jaundice	"	"
5	107	0.32	43.6	98.8	Delayed	3.3 "	Portal cirrhosis liver—ascites	Necropsy	No jaundice
6	97	2.46	38.2	92.6	"	Positive	"	Clinical	"
7	79	0.0	22.8	55.8	"	"	"	"	"
8	72	0.0	41.6	57.5	Negative	Negative	Syphilitic cirrhosis liver—polyserositis	"	"
9	57	1.22			Delayed	Positive	Syphilitic cirrhosis liver—ascites	"	Infantile—no jaundice
10	142	3.703	49.8	79.8	Immediate	8.67 units	Biliary cirrhosis (non obstructive)	"	Jaundice present—no ascites
11	157	3.36		82.6	"	15.795 "	Salvarsan jaundice—no ascites	"	"
12	153	3.29	44.2	71.4	"	6.605 "	Syphilitic cirrhosis liver	"	No ascites—jaundice present

KALA-AZAR IN MADRAS AND ITS BEARING ON EPIDEMIOLOGY OF THE DISEASE IN INDIA

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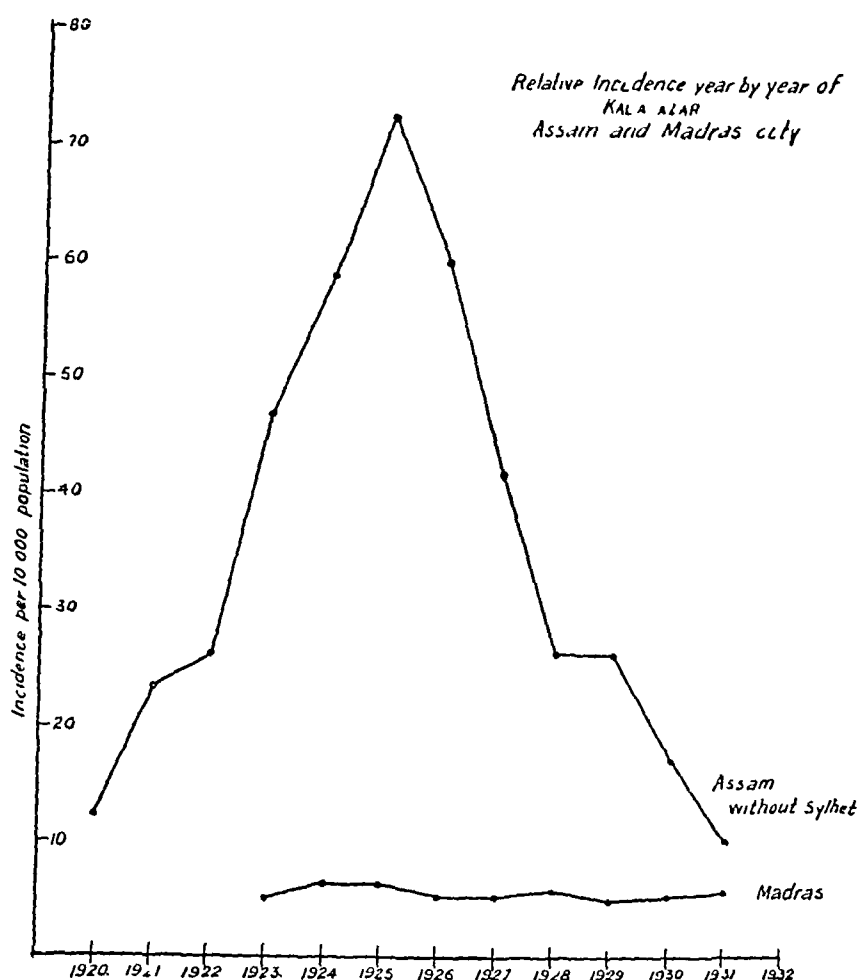
EARLY HISTORY

THE history of kala-azar in Madras virtually commenced in 1903 with the discovery of the parasite which causes the disease. There is little doubt that, if the records of the hospitals were searched, many instances of 'chronic malaria' in which quinine had no action could be found, but the specific clinical syndrome was not generally recognized and, though a few clinical observers, as for example Donovan, recognized a definite group of cases of persistent splenomegaly and suspected a different ætiology, no differentiation of this group was attempted (there are still many conditions which resemble kala-azar clinically but which are not due to leishmania infection) and it was not associated with the specific syndrome 'kala-azar' which had been recognized in Assam many years before.

The incidence of a hundred or even two hundred and fifty—taking the present-day figure—cases of an ill-defined disease in a community of over half a million does not make medical history or even attract the attention of the sanitarian, and this absence of any definite history of kala-azar in Madras before 1903 must not be accepted as evidence of the non-existence of the disease prior to this date. In other parts of India there is a very different story to be told. The epidemic which swept through Assam in 1875 attracted a considerable amount of attention, not only of the sanitarian but of the Government whose revenues were seriously affected. It was recognized as a distinct disease and given the name *kala-azar*. In the same way in Bengal and Bihar the disease was recognized long before the specific parasite was demonstrated.

Chart 1 shows the number of cases of kala-azar treated year by year in Assam province (excluding Sylhet) since 1920 and in Madras city during the period of the last survey, relative to the populations, on the basis of the Assam population being roughly 5.5 millions and Madras city's roughly half a million, throughout the whole

CHART 1.

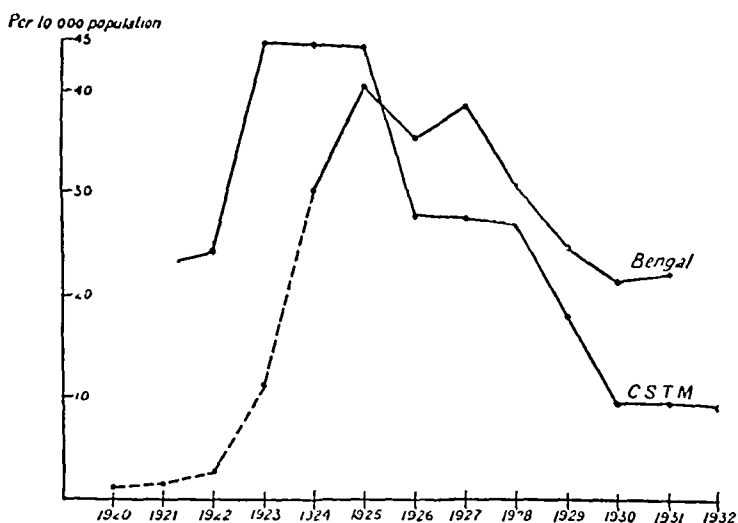


period Chart 2, drawn to the same scale, shows the number of cases treated year by year in Bengal since 1920 and in the kala-azar out-patient department of the Calcutta School of Tropical Medicine since 1922, relative to the population, on the basis of a constant population of 46.5 millions in Bengal and on the assumption that the School draws its patients from a population equal to one-quarter of that of Calcutta, or roughly a quarter of a million.* The sharp rise and equally sharp fall

* Many of our patients come from outside Calcutta, and there are four other large hospitals in the city and a number of smaller ones, so it will be seen that this figure is purely arbitrary, but it serves the purpose as it is only the incidence from year to year that is being contrasted

in the Assam curve stands out in marked contrast to the more or less straight line of Madras. As we have suggested above, the only figures available for kala-azar in Bengal are probably unreliable, especially with regard to rise in the incidence curve and it will be safer to consider only the second half of the curve. It will be seen that the fall in the Bengal curve occurs at the same time as the fall in Assam curve, but that it is not so sharp and that it ceases a long way from the zero line.

CHART 2



Relative incidence year by year of kala azar in Bengal, as shown by the Bengal provincial returns and the attendance at the Calcutta School of Tropical Medicine

Conclusion—It is thus apparent that in Madras the disease is truly endemic and not subject to much variation from year to year, in Assam it is epidemic and disappears almost entirely from many places in the inter-epidemic period, in Bengal, the state of affairs is intermediate between these two extremes, it is endemic, more or less throughout the province, but is subject to periodic increases of incidence. Or—to refer to the disease of a population as one would the disease of an individual—in Madras it is chronic, in Bengal sub-acute with periodic acute exacerbations, and in Assam it is acute with periodic relapses.

Kala-azar distribution in the city—It is not possible, as Cunningham and Varadarajan (*loc cit*) pointed out, to compare accurately their report with that of Korke (*loc cit*) as there was a re-arrangement of divisions in Madras between the times of these two surveys. If one takes the six divisions which provided the largest number of cases in the first survey it will be seen that they correspond roughly with the six most heavily infected divisions in both the second and the third surveys. Cunningham and Varadarajan consider, however, that there is definite evidence of a centrifugal spread of the disease from its focus in George Town, their figures provide some evidence of this. They say, 'The central endemic area is

the same in both, and Korke mentions cases as coming from every one of the areas where cases have been recorded in our survey. He states that the chief endemic focus of kala-azar roughly speaking is restricted to an area whose radius is nearly two miles and whose centre is the heart of George Town. We believe that this state of affairs no longer exists. The disease appears to have advanced to the extent that there is evidence that certain of these outlying foci are themselves new areas of infection. The outer fringe of infection has also apparently extended.

There is a general impression amongst medical men in Madras that this centrifugal spread of the disease is still continuing. Actual figures do not, however, entirely support this impression. As the number of cases in the present survey are about three times those of the previous surveys we cannot compare the actual numbers of the cases observed, but if we reduce the figures in each survey to percentages of the whole of that survey, we shall see that there is a remarkable similarity in the relative distributions in the two surveys (Table II). The six most heavily

TABLE II

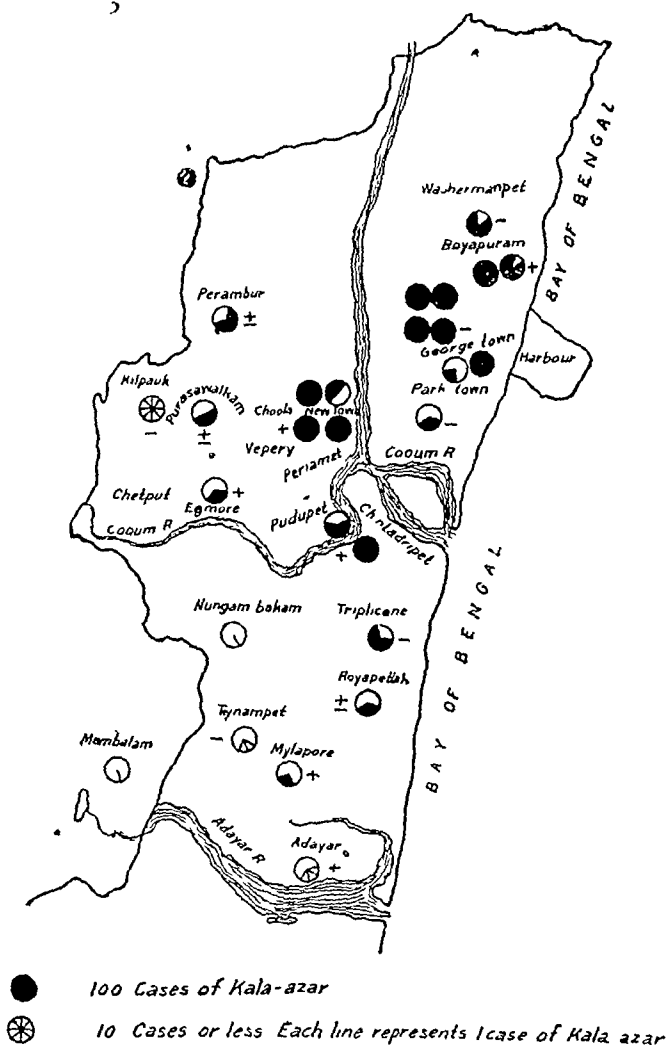
Locality	SECOND SURVEY		THIRD SURVEY		Relative increase or decrease
	Number	Percentage of all cases	Number	Percentage of all cases	
George Town	215	41.11	522	33.38	—
Choolai, New Town and Vepery	79	15.11	311	19.96	+
Royapuram	38	7.27	188	12.06	+
Chintadripet and Pudupet	38	7.27	142	9.11	+
Washermanpet	28	5.35	75	4.81	—
Triplicane	34	6.50	68	4.36	—
Perambur	21	4.02	60	3.85	±
Purasawalkam	14	2.68	46	2.95	±
Park Town	18	3.44	38	2.44	—
Royapettah	11	2.10	35	2.24	±
Egmore	6	1.15	31	1.99	+
Mylapore	7	1.34	24	1.54	+
Kilpauk	4	0.76	9	0.58	—
Teynampet	6	1.15	3	0.19	—
(Adayar)	0		4	0.26	
Mambalam	0		1	0.06	
Nungambakam	0		1	0.06	
(Tondearpet)	4	0.76			
TOTALS	523		1,558		

infected divisions are the same in each, together the cases in these constitute 82.61 and 83.68 per cent of the whole in the second and third surveys, respectively. Admittedly, in the recent survey there is a relative decrease in the George Town

figures, but of the outlying divisions only Egmore and Mylapore show a slight, a very slight in the latter case, relative increase, and Adayar, which was not mentioned in the previous survey and was presumably free, has provided 4 cases (see Map of Madras)

MAP OF MADRAS,

Showing the distribution of Kala azar cases in the different divisions of the City
(1923-1931)



On the whole it can be said that there is little evidence of any change in the relative distribution of the cases amongst the various divisions in Madras, or of any shifting of the centre of the endemic focus during the last 9 years

KALA-AZAR OUTSIDE MADRAS CITY

Cunningham and Vaiadarajan in their survey reported 57 cases as coming from outside Madras city, the figures for the present survey are 115. A complete history was seldom available in these cases, so that the locality where infection occurred is not usually definitely ascertainable, but the frequency of the appearance of these cases makes it fairly certain that there are endemic foci of infection dotted about the plains of Southern India, more especially in the east coastal areas, and from the records of the recent survey 12 cases, or 10 per cent of the outside cases, are reported to be from areas on the west coast, which has hitherto been believed to be free from kala-azar. Some of these foci of infection have been discovered and there is little doubt that if more careful investigation were carried out others would be found. This, of course, could not be done in the time available for the recent survey. Instead, the areas which were known to be endemic and had been surveyed by the junior writer (K. V. K.) 8 years previously (Turkud, Krishnan, and Iyer, 1926) were investigated.

The most important of these is Kayalpatnam, an isolated village community on the east coast some fifty miles north of Cape Comorin. Here in 1924 out of 109 cases of enlarged spleen examined 82 were found to be cases of kala-azar, in the recent survey 77 cases were examined and 46 were diagnosed kala-azar. It is thus apparent that the state of affairs is much as it was 8 years earlier, a slightly lower percentage of cases—60 per cent as compared with 75 per cent—proved to be kala-azar, but the difference is not great. In the same way in the other places visited conditions had undergone little change with the single exception of Rameswaram island which will be referred to again.

The inhabitants of Kayalpatnam say that the disease is not new. There is evidence of its existence 40 to 50 years before the time of the first survey of this village in 1924, a number of elderly inhabitants had the remains of calcareous ovis, which had followed an attack of fever with splenic enlargement when they were children.

On Rameswaram island not only was there found to be a considerable reduction in the incidence of enlarged spleen but also the percentage of kala-azar amongst these was distinctly less—17 per cent compared with 62 per cent—in the last investigation.

There has been a considerable reduction in the incidence of malaria in this island during the last few years. The Director of Public Health, Madras, has kindly provided us with figures which show that the spleen rate in Rameswaram was reduced from 11.0 per cent in 1929 to 2.2 per cent in 1931. Marked reduction in the spleen rate have also been noted in Pamban and Mandapam. This observation is of interest as it supports a theory put forward by the writers that one of the main predisposing causes of kala-azar in rural areas is malaria. Rameswaram is the only area in the Madras Presidency where we have been able to show that kala-azar is diminishing in incidence. No special measures have been instituted against kala-azar, but measures against malaria have been successful not only in reducing the malaria but also in reducing kala-azar.

Thus, observations outside Madras city indicate that in the majority of the rural endemic areas also there is little evidence of any change in the incidence of the

disease from year to year. In some of them there is evidence of the existence of the disease for at least 50 years.

THE EFFECT OF TREATMENT ON KALA-AZAR INCIDENCE

In Madras city there is little evidence of any change in the kala-azar incidence during the last 30 years. The available evidence suggests that, if there has been any change at all, there has been an increase rather than a decrease during the last 12 years since effective treatment was introduced. In Kayalpatnam the results of treatment have been so unsatisfactory as to be almost negligible and here also there has been no change in the incidence. Thus, in one place treatment has been effective as far as individual kala-azar patients are concerned, and in another place treatment has been highly unsatisfactory, yet in both places the effect on the incidence of the disease in the general population has been negligible. The obvious conclusion is that in Madras the disease cannot be controlled by treatment of the cases only.

THE DIFFERENT CLINICAL TYPES OF KALA-AZAR

Little has been written regarding the difference in the clinical type of kala-azar case encountered in different localities in India. Rogers (1910) drew attention to the difference between the more acute form of the disease which he saw during the epidemic periods in Assam and the more chronic type of case usually seen in Bengal and Sylhet (politically Assam but physiographically like Bengal), though he stressed the many points of similarity between these two types. Shortt (1932) refers to the acute fulminant type as being characteristic of the years of the peak of the incidence curve in Assam and rarer since the decline of the epidemic. In our experience in Bengal we have encountered all the clinical types, the rare fulminant type in which the patient dies after a few months' illness of hyperpyrexia and hæmorrhages, the typical acute type in which the fever is intermittent or remittent but continues to rise daily to 103°F or so for many months on end—the patient is thin and emaciated and the spleen is large, the chronic type, clinically very like chronic malaria, the patient often giving a history of illness for a year or so with only occasional bouts of fever, and attending first for some complication, such as dysentery or pneumonia, and finally the real ambulatory type, the well-developed patient who seldom suffers from fever but who has had an enlarged spleen which does not worry him much and who would normally not seek medical advice. The second and third types form the bulk of our patients in Calcutta, we have not observed any difference in the proportions of the two types at the peak of the hyper-endemic waves, as compared with the present time. The last-mentioned type is not often seen by us in Calcutta but this does not necessarily mean that it is rare, it may simply mean that these patients do not come to our notice. Our own experience in Assam during the height of the epidemic wave was very short, but from our limited experience during the epidemic period, from our more extensive experience in recent years, and from reports we have seen, we are satisfied that all four types occur, both during the epidemic and during the non-endemic periods, but that during the non-epidemic

period there is a distinct swing to the right, and as Shortt has said there are fewer cases of the fulminant and acute types

In Madras all types seem to be represented but in our experience the ambulant type is far more common than it is in Assam or Bengal—in Kayalpatnam a number of these cases were encountered both during the last survey and during the present one, and the fulminant type is very rare. In Madras city the acute type is the rule but the chronic type is also seen fairly frequently. No periodic change in the type of case has been noted.

Thus, it can be said that there is no real difference in the clinical picture of kala-azar seen in the three areas of its activity, or between the epidemic and non-epidemic periods, but that there may be, and probably is, a difference in the proportions of the various types in these different areas, and that in Assam the relative incidence of the different types varies at different times in relation to the annual incidence of the disease in the province.

Dermal leishmaniasis

Hitherto very few cases of dermal leishmaniasis (post-kala-azar) have been reported from Madras. We have been able to find only 4 cases in the literature. In Bengal the condition has been recognized for more than 10 years, it is now very frequently encountered, and its protean skin lesions have been described and depicted very frequently in medical journals so that one would expect that most medical officers would be familiar with this condition. Nevertheless we felt that this rarity of the condition in Madras required further confirmation before being accepted as a fact.

Investigations in Madras soon showed that we had obtained a false impression regarding the rarity of these lesions. It was found that 14 cases had been diagnosed in Madras in the previous two years, these were all typical cases and there seems little chance of any mistake having been made in diagnosis as this had been confirmed in most instances by the finding of the parasite. The cases that we were shown all had typical and well-developed lesions.

It was felt that personal investigation into the incidence of this disease should be reserved for the rural foci where more intimate contact with the general population is possible. In Kayalpatnam five cases were found during a very short visit. They were all well-developed and clinically-typical cases, four of them had been diagnosed kala-azar and had been treated for this disease. Amongst the same population 46 cases of kala-azar were discovered. Undoubtedly many cases of both conditions were missed, but the patients suffering from kala-azar are likely to come forward more readily than those with dermal lesions—especially the early lesions which are often unnoticed by the patients themselves, the proportion of dermal cases to visceral cases in this village is thus certainly not less than 1 to 9. Compare this with the relative incidence of dermal leishmaniasis and kala-azar in the village area in Bengal which the senior writer (I. E. N.) investigated (Napier, 1931). In this area the population was about 5,000, the kala-azar incidence was 387 and the dermal leishmaniasis incidence 10, over a period of six years. By systematic examination of 120 persons who had had kala-azar, 6 were found to have dermal lesions. If we take the first figures, we see that the proportion of dermal to visceral

cases was 1 to 39, or if we take the other figures it was 1 to 20. Furthermore, most of these Bengal cases only showed early lesions which would not have been recognized by anyone who had not had considerable experience in diagnosing this condition in all its stages—in four cases the patients themselves had not noticed the lesions—whereas the Kayalpatnam cases all had well-developed nodular lesions.

It is apparent that in this village at any rate the dermal lesions are more common than they are in Bengal generally. Whether this remark is applicable to the whole of the Madras endemic areas is not certain, but it seems very probable that, as the knowledge regarding the diagnosis of this condition is disseminated amongst medical practitioners in Madras and Southern India, more and more cases will be diagnosed.

Again, in Assam dermal leishmaniasis is apparently rare. There is every reason to suppose that this rarity is not only apparent but real, as during the whole six years the Kala-azar Commission were working in Assam not more than 5 or 6 cases were encountered. The officers on this Commission were all familiar with this condition, they visited many parts of the province and saw tens of thousands of cases of kala-azar.

The weight of evidence thus suggests that the proportion of dermal to visceral leishmaniasis is very high in Madras, not so high in Bengal and very low in Assam, that is to say, the incidence of dermal leishmaniasis is in direct proportion to the 'chronicity' of the leishmania infection in the population.

DISCUSSION

The observations recorded in this paper appear to us to have some importance in their relationship to the general problem of the epidemiology of the disease in India. The writers (Napier and Krishnan, 1931) have already suggested that the dermal lesions, the sequelæ of the visceral infection, may play some important part in this connection.

Dermal leishmaniasis may be considered as a phenomenon of imperfect immunity response on the part of the host. We will leave out of consideration for the time being the effect of treatment. Amongst those who survive the visceral infection, there will be a percentage that show evidence of this imperfect immunity response and develop dermal lesions. When the disease is invading virgin soil, the mass immunity is low, the infection is easily established, and the disease is epidemic, the clinical disease—the evidence of the fight between the parasite and host—is acute, there is little scope for host-parasite adjustment, and the result is a decisive victory on one side or the other. This was the state of affairs in the early Assam epidemics, history relates that 95 per cent of patients died, the rest being completely cured. Amongst so small a number of surviving hosts, there would be a negligible number, if any, of examples of imperfect immunity response.

With an increase in mass immunity in the population, which inevitably follows the subjection of a population to repeated epidemics, the disease will become endemic, it will assume a less acute symptomatology—the host-parasite battle will be less severe, there will be more scope for host-parasite adjustment, there will be a greater number of surviving hosts, and the examples of imperfect immunity

response will begin to appear. This is the state of affairs that existed in Bengal up to about 15 years ago.

With the further increase in mass immunity the chronic forms of the visceral disease appear, there are still more survivors and amongst these a greater number show evidence of imperfect immunity response, i.e., dermal lesions, this is the state of affairs which we believe exists in some rural endemic areas in Southern India to-day.

Biologically, the final goal of all parasitization is symbiosis. From the point of view of the parasite the visceral localization is very unsatisfactory as the result will be either cure, or the death of the host, it is not to the advantage of the parasite to kill the host, but circumstances lead to its doing so. The dermal lesion, a non-fatal condition which may last for many years, is a compromise, but the lesions themselves are evidence of some host resistance, and a state of affairs is conceivable where complete *symbiosis* occurs, the parasite existing in the skin without producing lesions. Actual experience suggests that this may occur as we have seen many cases in which lesions, so slight as to be overlooked by all but the most experienced, have existed for years.

We have referred to host-parasite adjustment, the implication is that the compromise is brought about by a change in both the host and the parasite. Though the evidence of any change in the parasite may be small it is not inconceivable that the parasite does take some part in a combined movement towards symbiosis. Thus, in Assam where the infection is transmitted from visceral cases there was little tendency for the transmitted infection to assume a dermal form, in Bengal with the appearance of the dermal forms an increasing number of transmissions occur from dermal cases—in the hypo-endemic periods they are possibly all from dermal cases—and there will be a greater tendency on the part of the parasite to assume a dermal localization.

We believe that specific treatment has the effect of aiding the natural immunity response. If this is the case the result of the introduction of specific treatment will not produce any sudden change in the nature of the disease though it will probably tend to accelerate the natural evolutionary adjustment between host and parasite. When the association is new and there is a severe balanced struggle the result of treatment in the majority of cases will be a weighting of the scales markedly in favour of the host. The actual number of cases of imperfect immunity response will be but few though greater than prior to the introduction of treatment in the locality. When, however, the association is older and there is a natural tendency towards compromise or symbiosis treatment merely increases this tendency and gives rise to a greater number of instances of imperfect immunity response. This we think is the reason why the proportion of dermal cases to visceral cases is higher in Madras than in either Bengal or Assam.

Our conclusion is that in Southern India are the oldest foci of kala-azar, as far as India is concerned, that here the disease has passed through the two stages now seen to exist in Assam and Bengal, respectively, and has reached a 'chronic' stage where there is no rise and fall in incidence from year to year and the disease is truly endemic, and that the transmission is almost entirely from dermal infections, so that treatment of kala-azar cases alone does not affect the general incidence of the disease.

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APPENDIX

REPORT ON INVESTIGATION IN MADRAS

BY

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IN November 1931 Dr Napier paid a visit to Madras and to some of the kala-azar areas in the Madras Presidency. In the report that he submitted he suggested that an officer from the Kala-azar Inquiry should be sent again to Madras to continue the work he had started, and he indicated the lines of investigation that would be most profitable. In accordance with this plan the writer proceeded to Madras on 1st April, 1932, and from 3rd April, 1932, to 7th July, 1932, he conducted a kala-azar investigation in Madras city, in Rameswaram island, Devipatnam and Kilakarai in Ramnad district, and at Kayalpatnam in Tinnevely district.

The chief object of the investigation was to note the changes that might have occurred in the incidence and character of kala-azar since the last survey in 1924, and to obtain a correct idea regarding the prevalence of dermal leishmaniasis in the places visited.

The scheme of work adopted for the Madras city was the same as that of Korke (1912) and of Cunningham and Varadarajan (1923), and consisted in a detailed analytical study of the kala-azar case records of the four large hospitals in the city, i.e., the General, the Royapuram, the Royapettah and the Women and Children's hospitals, for the period 1923 to 1931.

As the hospital records of the endemic areas outside the city of Madras were incomplete and unreliable, the plan of work adopted by Turkhud, Krishnan and Seetharama Iyer (1926) during their survey of these areas in 1924-1925 was followed. This consisted in the collecting together of all cases of enlarged spleens in one central place of the village visited and from among them picking out kala-azar cases after a careful clinical examination followed by an aldehyde test or demonstration of the parasite in the blood, or both.

KALA-AZAR IN MADRAS

Madras city — The Madras hospital records show that 2,253 cases of kala-azar were treated during the period 1923 to 1931, this excludes the cases treated in the General hospital in 1931 as records were not available. In addition to this, by interviewing private practitioners in some of the heavily-infected areas in the city, records were obtained of 229 cases that had been treated by them, but as the information regarding these cases is incomplete these are not being included in the statistical analysis presented below.

The yearly distribution of kala-azar cases in the four large hospitals in Madras city is given below in a tabular form —

TABLE

Distribution of kala-azar cases in the Madras hospitals from 1923 to 1931

Years	1923	1924	1925	1926	1927	1928	1929	1930	1931	TOTAL
General hospital	163	187	182	152	165	140	120	137	130*	1,376*
Royapuram hospital	73	112	97	82	60	108	82	89	107	810
Royapettah hospital	1	5	16	15	19	22	22	17	25	142
Women and Children's hospital	2	3	6	6	3	3	15	7	10	55
TOTALS	239	307	301	255	247	273	239	250	272*	2,383*

* The figure for the General Hospital for 1931 is estimated as records were not available at the time.

From the above table it will be seen that the number of cases of kala-azar admitted into the four large hospitals in the city has been more or less constant during the last nine years.

In a detailed study of the kala-azar case histories only 1,558 of the 2,253 cases of kala-azar that were treated in the hospitals could be associated with any definite place of residence within the city. The distribution of these cases according to the municipal divisions from which they originated is shown in a tabular form (*vide* Table II of the paper). It will be evident that there are at least 3 chief foci for kala-azar in Madras city, one in George Town and Royapuram, another in Choolai and New Town, and a third in Chintadripet. These three have always been and still are the worst areas. Even from the divisions farther removed from these foci one or more cases are being reported every year. There is a very strong local

impression that the disease is slowly and steadily spreading in all directions and involving new areas in the city, but the evidence adduced does not support this

KALA-AZAR CASES FROM OUTSIDE THE CITY OF MADRAS

Of the remaining 695 kala-azar case records studied, 580 were incomplete, and the other 115 showed that the cases originated from outside the city of Madras. Out of these 105 came from known kala-azar endemic areas and the other 10 from Malabar which has hitherto enjoyed the reputation for being free from kala-azar. One is not certain whether all these ten patients actually contracted the infection during their residence in Malabar, it is possible that some of them may have been infected from endemic areas that they had visited previously, the histories are not clear on this point. In addition to the above ten cases I learnt of two more that a practitioner in the city had treated. Both these cases had come from Calicut, one was a girl of 6 and the other a boy of 10. On interviewing relatives of these patients it was found out that the boy for certain had never left Calicut for any other endemic area prior to his illness, but that the girl had paid two visits to Madras a year previously and on both these occasions she had stayed in Royapettali. The names of places in Malabar from which the above-mentioned kala-azar cases possibly contracted the infection, are Ponnani (1), Palghat (2), Badagara (1), Mangalore (2), Travancore (1), Calicut (2), the exact place of residence of the other case not being noted.

Diagnosis—The methods adopted for the diagnosis of kala-azar cases in the hospital are given below in a tabular form. The figures represent the number of cases diagnosed by each of the methods—

I Discovery of parasite in

Peripheral blood	{ smears cultures	{ 68 4	} 736
Liver puncture		242	
Spleen puncture		422	

II Serological tests	{ Aldehyde Chopra's antimony test	{ 690 1	} 691

III Clinical		826
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In Cunningham and Varadarajan's series only 20.5 per cent were diagnosed by the finding of the parasite. There is no reason to believe that diagnostic inaccuracy will have vitiated the figures to any great extent in either survey.

It will be evident from the table that Chopra's antimony test is not very popular with the physicians in hospital. Inquiries of private practitioners showed that they find the test of greater value than the aldehyde test for the diagnosis of early cases of kala-azar. If it is so, then the test well deserves a more extensive trial in hospitals.

Treatment—Urea-stilamine is still the most popular remedy. Neostibosan (or Bayer '693'), which is now widely used in Bengal and Assam and almost exclusively at the Calcutta School of Tropical Medicine and outside India, has not as yet been given a fair trial.

Mortality—The mortality rate among the kala-azar cases treated in hospital was 4.1 per cent (93 out of 2,253).

In the following tables the incidence of the disease according to age, sex and religion is given —

Age —

1 to 5 years	69 cases	3.45 per cent
6 to 10 "	238 "	11.91 "
11 to 15 "	363 "	18.17 "
16 to 20 "	428 "	21.43 "
21 to 25 "	365 "	18.27 "
26 to 30 "	203 "	10.16 "
31 to 35 "	101 "	5.06 "
36 to 40 "	98 "	4.91 "
Over 41 "	132 "	6.61 "

TOTAL 1,997 cases

Sex —

Males	1,677
Females	576

TOTAL 2,253

Religion —

	Hindus	1,339 cases
Christians	{ Anglo Indians	343 "
	{ Indian Christians	203 "
	Mohammedans	122 "
	Other castes	246 "

TOTAL 2,253 cases

From the above tables no useful inferences can be drawn as the figures are those of a selected hospital population and, as such, subject to various limitations and fallacies. They compare very closely with the figures of the last survey except that the proportion of Indians is considerably greater.

KAYALPATNAM

This is a sea coast union village in the Tinnevely district, with a population of nearly 9,000. The last kala-azar survey of the place was conducted by the writer in 1924. At that time 109 cases of enlarged spleen were seen and of these 82 proved to be kala-azar. During this survey 77 cases of enlarged spleen came up for examination and of these 46 turned out to be kala-azar. This number does not, however, give a correct picture of the incidence of the disease in the place. From personal observations as well as from a study of the dispensary records it is thought that there are at present at least a hundred cases of kala-azar in Kayalpatnam. The conditions as regards kala-azar do not seem to have changed at all. The treatment offered in the dispensary is, as it has always been, anything but satisfactory. The medical officer complains that he does not get any of the newer remedies, such as urea-stibamine, for use, he is still being supplied only with solution of antimony tartrate, and with this his cure-rate is never very high. The people are mostly very poor and cannot afford to buy urea-stibamine, or other efficient remedies. In a closed Mohammedan community, such as the one at Kayalpatnam, and with a limited number of kala-azar cases, it would be interesting to see if the disease could be completely eradicated by correct diagnosis and intensive treatment of every

case of kala-azar. Such a campaign would be neither too costly nor too difficult to carry out, and the result obtained should be valuable from several points of view.

Phlebotomus argentipes IN KAYALPATNAM

One of the most interesting discoveries made during this visit to Kayalpatnam was the finding of *P. argentipes* in the place. It will be remembered how during the previous survey despite a determined effort to find sandflies no success resulted. Although the failure to obtain sandflies was at the time attributed to the unsuitability of the season and such other causes, the opponents of the sandfly theory have always tried to make capital out of this. The finding of *P. argentipes* at Kayalpatnam proves finally that in every kala-azar endemic area in India these sandflies are prevalent.

Among the sandflies caught, three species were represented, i.e., *P. argentipes*, *P. minutus* var., and *P. malabaricus*.

RAMESWARAM ISLAND

Since 1906 this island has persistently enjoyed a reputation for being a badly infected area for kala-azar. During the last survey of the island in 1924-1925, 77 cases of kala-azar were discovered. This time the writer went there with the full expectation of finding a good number of kala-azar cases, but even after a thorough search was able to get only 10 cases. Although it is not claimed that this figure gives a true representation of kala-azar incidence in the island it certainly helps to show that the disease is definitely on the decline. The inhabitants, as well as the medical officer of health, are quite positive that cases of enlarged spleens are fast disappearing from the island. What the true cause of this decline is one is not certain. But it is suggested that the intensive anti-malarial operations that are now being carried out in the island are to some extent responsible for this decline in kala-azar.

The following table gives the number of kala-azar cases detected in each of the places visited —

TABLE

Showing the number of kala-azar cases detected in some of the kala-azar endemic areas in the Madras Presidency

Name of village	Name of district	Number definitely diagnosed as kala azar	Number probably kala azar	Number definitely diagnosed as non kala azar	Total number of cases seen
Kayalpatnam	Tinnevely	21	25	31	77
Kilakarai	Ramnad	20	18	14	52
Devipatnam	"	3	1	3	7
Rameswaram island	"	7	3	49	59
TOTALS		51	47	97	195

DERMAL LEISHMANIASIS IN MADRAS

A study of the hospital records, as well as inquiries of practitioners, showed that from September 1930 to April 1932, 14 cases of dermal leishmaniasis had been diagnosed in the city of Madras. Of these, three that were being treated at the time of the writer's visit were shown to him. There was no doubt that they were typical cases.

With regard to the kala-azar endemic areas outside Madras city no case of dermal leishmaniasis has hitherto been reported from any of them. During the visit to these areas therefore the writer was especially on the look out for dermal cases. Inquiries showed that the medical officers of these areas had never seen a case of dermal leishmaniasis before and could not even recognize one as such when they saw it.

In Kilakarai a suspicious case of dermal leishmaniasis was brought, but as this patient did not turn up for a proper diagnosis one cannot be certain about this case.

In Kayalpatnam, it was an easy matter to obtain within a short period five cases of dermal leishmaniasis. In every one of them the clinical diagnosis was confirmed by the finding of *L. donovani* in the smears from the lesions. The distribution and nature of the lesions showed absolutely the same characteristics as in Bengal, depigmented patches, nodules and butterfly erythema were all met with. The part of the body most often affected was the face, lesions were generally found on the tip of the nose, on the chin, margin of the lips, and on the pinnæ of the ears. The disease is certainly not rare and if one is on the look out for it he is sure to obtain a few cases at any time of the year. In the past the medical practitioners were not familiar with the protean manifestations of the disease, and often mistook the cases for those of syphilis, leprosy, and fungus infection. If arrangements could only be made to demonstrate dermal cases to the medical men in the kala-azar endemic areas and to teach them to diagnose the disease correctly then increasing numbers of cases will certainly be reported every year. There is no reason to believe that the conditions, responsible for the causation of dermal leishmaniasis in Bengal, are not prevalent in Madras.

The writer wishes to express his thanks to the Surgeon-General and the Director of Public Health, Madras, both of whom very kindly made all the arrangements necessary for the carrying out of the work he had on hand. His thanks are also due to the Personal Assistant to the Surgeon-General, the Superintendents of the hospitals in the city of Madras, the Professor of Pathology, Medical College, Madras, the Director, King Institute, Guindy, the District Medical Officers and the District Health Officers of the Ramnad and Tinnevely districts for the facilities and assistance that they willingly offered to him.

THE INFECTION OF *PHLEBOTOMUS ARGENTIPES* FROM DERMAL LEISHMANIAL LESIONS

BY

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SINCE the first observation of leishmania in the protean skin lesions that are a comparatively common sequel to generalized leishmania infection (kala-azar), there has been little doubt that the organism causing these lesions was the same as that which causes the visceral infection. No morphological differences have ever been demonstrated. Culturally it behaves like *Leishmania donovani* (Knowles, Napier, and Das Gupta, 1923) and when inoculated into a mouse's peritoneal cavity it gives rise to a generalized infection (Das Gupta, 1927), admittedly in a monkey localized nodular lesions were produced without a generalized infection, but this has been reported in animals inoculated with the visceral strain. A further point of similarity in the two strains was provided by Shortt, d Silva, and Swaminath (1928) who showed that *Phlebotomus argentipes* fed on a nodule developed a flagellate infection similar in every way to that developed in this fly when it is fed on a kala-azar patient. These workers fed the flies on the individual nodules by means of a test-tube inverted over the nodule.

About this time, that is in 1928, some of the writers repeated the last-mentioned experiment, but in addition they fed other flies on the depigmented areas in cases of post-kala-azar dermal leishmaniasis. The flies were not, except in a very few instances, fed a second time, they were dissected when they died, or at the end of the fourth day. One fly fed on a nodular lesion showed a flagellate infection, but all the flies fed on the depigmented lesions were 'negative'. The number of flies dissected in this experiment was 77.

TABLE

Case, serial number	Type and site of lesions	AFTER WHICH FEED DISSECTED								Total dissected		Days on which dissected	REMARKS
		1st feed		2nd feed		3rd and 4th feeds		Number	Positive	Number	Positive		
		Number	Positive	Number	Positive	Number	Positive						
I	Nodular, arms and forearms	85	7	7	1	12	3	28	4	7th and 9th, 10th and 10th	*4th feed		
II	Depigment, lesions only, on thighs	32	2			5 3	2 2*	8	4	7th and 9th, 13th and 13th			
III	Advanced depigmented areas in the forearms	93	Several	3	1	25	6	57	8	Not noted			
	TOTALS	210		10	2	45	13	93	16				
	Percentage positive			20	2.83	28.89		17.20					

Some time later at a more favourable time of the year (i.e., during August-September) at the suggestion of the senior writer (L E N)—though actually during his absence—the two junior writers (C R D G and S M) repeated this experiment, but instead of dissecting the flies at an earlier date they allowed them to feed a second and, if they survived, a third and even a fourth time. The feeds subsequent to the first were on uninfected mice. On the nodules the flies were fed from a test-tube, but for the depigmented lesions the whole limb was put into a sandfly feeding cage and the flies were allowed to feed where they wished. The results of these experiments are summarized in the Table, cases I and II.

This particular experiment was not repeated until 1932 when there was a temporary shortage of kala-azar patients suitable for infecting sandflies. There was at this time in hospital a patient who had very extensive dermal leishmaniasis lesions all over his body, on his arms and forearms he had only depigmented lesions, but these involved nearly half the skin surface. Sandflies were fed on his arms on several occasions and a certain number dissected after subsequent feeds on uninfected animals. A high percentage of infected flies was found amongst these, the results of the dissections are shown in the Table (case III). A large number of flies were fed on this patient subsequently in our hamster infection experiments, but no separate record was kept of the results of dissection.

Finally, a number of sandflies were fed on the arms of a woman who had very early dermal lesions. These lesions were so indistinct that a senior member of the staff of this institution who was asked to examine her arms did not observe the lesions until they were pointed to him, and the woman herself was unaware of their existence. She had been treated by us two years previously for kala-azar and came to the hospital only to bring her child who had developed the disease subsequently. Unfortunately at the time this experiment was commenced the weather was becoming cold and flies were not feeding well, 20 flies were fed and were dissected after an interval of 3 or 4 days, of these one was found to be infected.

DISCUSSION

In a histological section of the granulomatous nodular lesion of dermal leishmaniasis, parasites will be seen lying in tissue histiocytes in the sub-papillary layer of the skin well within the reach of the proboscis of a sandfly. It is therefore quite natural that if a fly feeds directly on such a nodule that it will become infected. On the other hand, except by cultural methods, we have always failed to demonstrate the parasites in the depigmented lesions. It was therefore a matter of some considerable importance to decide whether or not sandflies fed on the skin exhibiting these lesions would become infected.

It will be seen that the infection rate amongst flies dissected within two or three days, that is before they took a second feed, was very low indeed. This is not very surprising, and is to be accounted for by the fact that the parasites ingested are few in number and will in many cases be overlooked when the sandfly is dissected. On the other hand if the flies are kept alive for some time the flagellates multiply and an infection will be recognized in almost every instance. It will be seen that the infection rate after the 3rd and 4th feeds was very high indeed, an infection rate of

29 per cent is higher than one usually obtains when the peripheral blood of a kala-azar patient is used as the source of the blood meal

It must be remembered that the flies in cases II and III were not placed directly on a depigmented area, but were at liberty to feed on any part of the skin that was exposed—usually a considerable portion of the limb, if not the whole limb. Many of the persons suffering from this condition have lesions all over the body, so that in Nature, when a sandfly feeds on such a person, the chances of that fly becoming infected are very considerable.

We have shown elsewhere that persons with obvious lesions are common in areas where kala-azar is endemic. All stages of development of the lesions are encountered, but the earliest clinical stages never appear within a year of the cure of the visceral infection. Now the distribution of the parasites to their dermal foci can only have taken place during the visceral infection and therefore they must have been latent for at least a year before they produced clinical lesions. We have shown above that sandflies can be infected from the very earliest clinical lesions and it is therefore quite possible that they can also become infected from a person before he begins to show dermal lesions.

Sporadic attempts have been made from time to time to find infected sandflies in Nature, but workers in India have never felt justified in spending much time on this investigation. Sandflies required for maintaining the stock of laboratory-bred flies are most easily caught in cow-sheds and there is often a certain amount of opposition encountered when the insect collectors try to collect flies from houses or huts. However, as early as 1926 Craighead (Shortt, Barraud, and Craighead, 1926) found a wild sandfly infected with a flagellate (number examined not stated) indistinguishable from *L. donovani*, and in 1928 in Assam the second writer (R. O. A. S.) found 7 infected flies out of 229 examined. About the same time the senior writer (L. E. N.) who was at that time in-charge of both the Kala-azar Commission in Assam and the Inquiry in Calcutta instituted a similar investigation in Calcutta. The device of feeding the flies on a mouse to prolong their lives was resorted to and 48 flies of which 40 were given two, and 8 more than two additional feeds on clean animals were dissected, one was found infected. In each case except the last-mentioned the flies were caught in huts where kala-azar patients were known to be living at the time.

It seems important that this particular line of research should be continued, as the finding of a number of infected flies in an endemic area during a hypo-endemic period would have a very obvious significance. A large number of flies would have to be dissected and the value of the work would be enhanced considerably if it were continued throughout a whole year. The positive finding in the last series of dissections was in a fly caught at the end of April, that is to say in the hot weather, in neither of the papers referred to above is the time of year when the infected flies were caught noted.

The possibility of dermal lesions constituting a reservoir for carrying the infection over a hypo-endemic period in the kala-azar-endemic areas has been discussed fully elsewhere (Napier, 1931, Napier and Krishnan, 1931, 1933). That the persons exhibiting skin lesions are an abundant source of infection for sandflies, we have shown above, and the suggestion is again put forward that this source of infection may not be limited to persons showing obvious clinical lesions.

SUMMARY

A flagellate infection rate of 30 per cent was demonstrated in a group of sandflies (*P. argentipes*) fed on the limbs of patients exhibiting the depigmented lesions of post-kala-azari dermal leishmaniasis

This high percentage was only achieved by giving the sandflies two or three subsequent feeds on clean mice, and thereby keeping them alive for some days, only one out of 38 flies not given a subsequent feed was found infected

A sandfly was infected from the arm of a patient who exhibited lesions so rudimentary that they would only have been noticed by a medical man who had had considerable experience with this condition. The possible significance of these observations is discussed

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THE GOITROGENIC ACTION OF SOYA-BEAN AND GROUND-NUT

BY

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[Received for publication, March 20, 1933]

The experiment.

FOUR groups of young albino rats were fed as follows —

- GROUP I (Controls) On a diet consisting of starch 60 parts, meat residue 20 parts, olive oil 10 parts, dried yeast 5 parts, cod-liver oil 2 parts, salt-mixture (McCollum's), containing 0.45 g of KI, 5 parts and distilled water *ad libitum*
- GROUP II On the same diet as above, but in which the cod-liver oil and yeast were replaced by soya-bean in the proportion of 1, per rat per day
- GROUP III On the same diet as above, but in which the olive oil, yeast and cod-liver oil were replaced by 15 parts of *unhusked* ground-nut
- GROUP IV On the same diet as above, but in which the olive oil, yeast and cod-liver oil were replaced by 15 parts of *husked* ground-nut

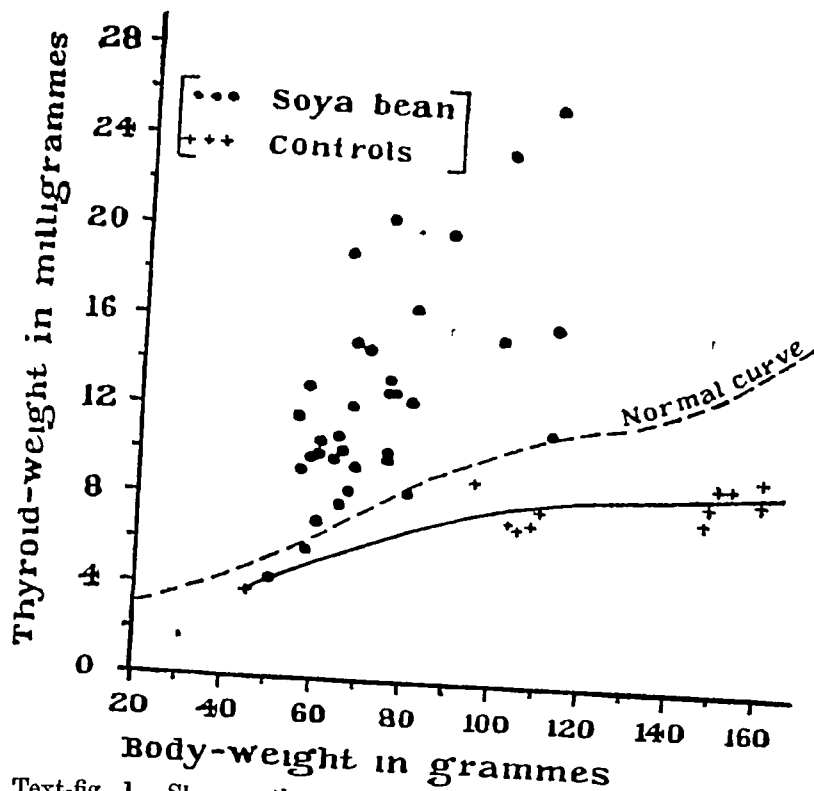
Each animal was confined in a separate screened cage under conditions of scrupulous cleanliness. The duration of the experiment was 91 days.

Results of the experiment

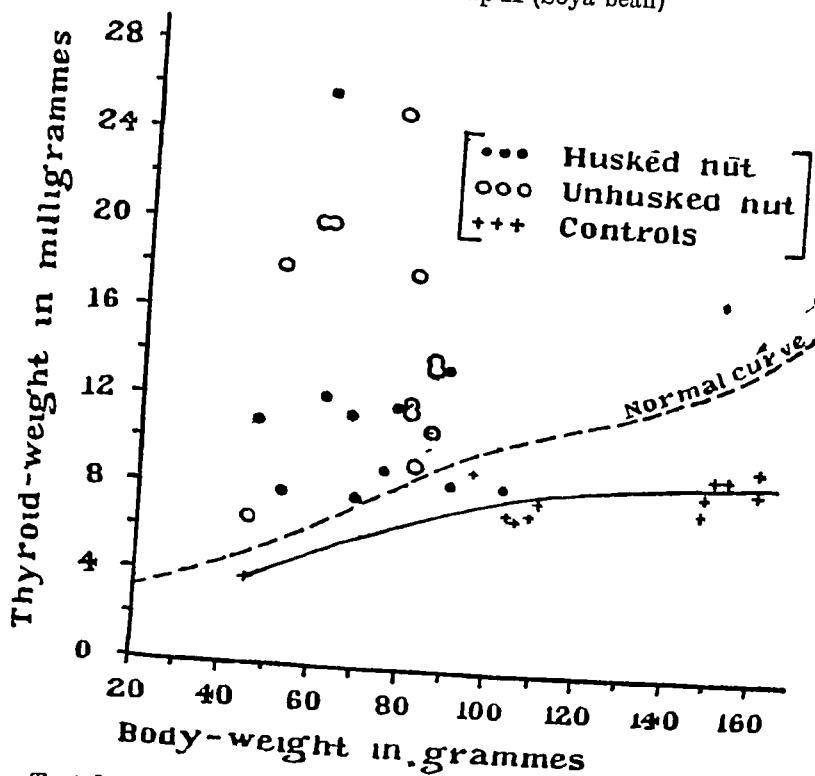
The results of this experiment are shown in Text-figures 1 and 2. From these it will be observed that a high incidence of thyroid enlargement occurred in Groups II, III and IV, while no cases of enlargement occurred in Group I (Controls).

The mean size ('r' = the mean thyroid-weight divided by the mean body-weight) of the thyroid gland in the four groups was as follows —

Group I (Controls)	6.63 ± 0.37	mg
Group II (Soya-bean)	17.2	'
Group III (Unhusked ground-nut)	21.69 ± 2.79	'
Group IV (Husked ground-nut)	17.35 ± 3.37	'



Text-fig 1. Showing the weights of the thyroid gland in Group I (Controls) and in Group II (Soya bean)



Text-fig 2 Showing the weights of the thyroid gland in Group I (Controls) and in Groups III (Unhusked ground nut) and IV (Husked ground-nut)



Fig 1



Fig 2.



Fig 3



Fig 4

Figs 1 to 4 Showing the histological appearances of the goitres produced in Groups II (Soya-bean) III (Unhusked ground-nut) and IV (Husked ground nut) Note keratinization cyst in Fig 2

The differences in size of the gland between that in the Controls and that in Groups II, III and IV are significant. They indicate that in the absence of a sufficiency of vitamin-bearing food-ingredients, both soya-bean and ground-nut were goitre-producing.

It will be noted that thyroid enlargement occurred despite the ingestion of iodine, as potassium iodide, in large amounts. The average food consumption per rat per day was 10 to 12 g. The amount of iodine ingested, as KI, was, accordingly, from 1,725 to 2,070 γ per rat daily. It has been shown in another place (McCarrison, 1930) that iodine may have a goitrogenic action when administered to rats fed on diets deficient in vitamin-bearing ingredients.

The histological appearances of the enlarged glands are shown in Plate VII, figs 1 to 4. The aberrant hyperplasia and keratinization of the glandular epithelium are characteristic of vitamin A-deficiency (McCarrison, 1927).

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EFFECT OF IODINE ON THE GROWTH AND METABOLISM OF THYROID TISSUE *IN VITRO*

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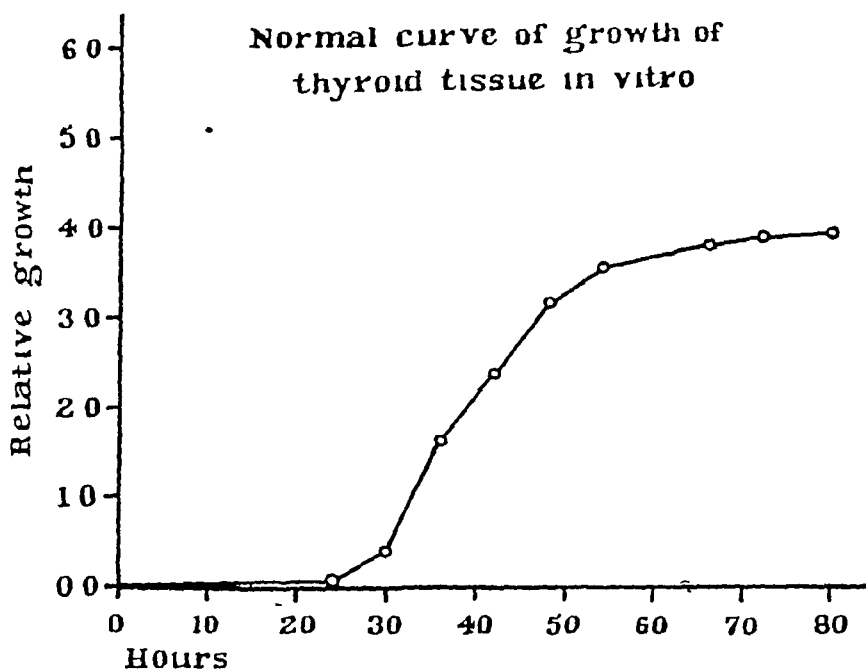
COVER-SLIP preparations of the embryo-chick (18-day) thyroid were made in the usual way, the medium employed being hen-plasma 50 parts, 9-day embryo-chick extract 25 parts, and Tyrode solution 25 parts. Thyroid tissue grows well in this medium, the rate of growth is indicated in Text-fig 1*. Growth during the first 54 hours is both epithelial—in the customary sheets (Plate VIII, fig 1, and Plate IX, figs 3 and 4) and fibroblastic (Plate XI, fig 8). Metabolic activity is marked, and from about the sixtieth hour onwards liquefaction of the medium occurs (Plate VIII, fig 1). When this liquefaction has reached a certain point large, single or multiple vacuoles appear in the mass of growing tissue, the final appearance ('Signet-ring stage') at about the eightieth hour being such as is represented in Plate VIII, fig 2. Thereafter growth ceases, owing to the accumulation of metabolites, and is resumed only after washing in Tyrode and replanting. The appearance at this stage is similar to that seen in cultures of Rous-Sarcoma—it is indicative of an intense metabolism of the growing thyroid cells.

Growth is largely dependent on the age of the plasma, the older the plasma the slower the growth.

For the purposes of the present investigation, observations were made at six-hourly intervals up to the eightieth hour, the growth being outlined on squared paper, with the aid of the camera lucida, and afterwards translated into curve-form (Text-figs 1 to 4). Means of estimating the effects of different concentrations of iodine in the culture-medium were provided both by the rate of the tissue-growth and by the rapidity with which liquefaction of the medium occurred.

* Relative growth = $\frac{B-A}{A}$, where A is the surface area of the original fragment and B is the final surface area of culture.

In the observations which follow iodine was added to the culture-medium in the form of sodium iodide. The salt was dissolved in Tyrode solution of standard composition, the solution being such that, when it comprised 25 per cent of the culture-medium, the amount of added iodine was 12.5, 25.0, 50.0 or 100.0 γ per 100 c.c. of the medium. In each observation several specimens were put up

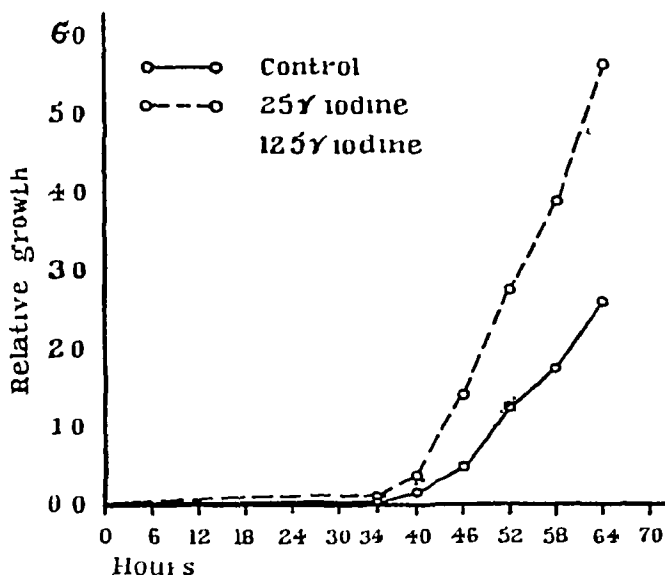


Text fig. 1 Showing normal curve of growth of thyroid tissue *in vitro*

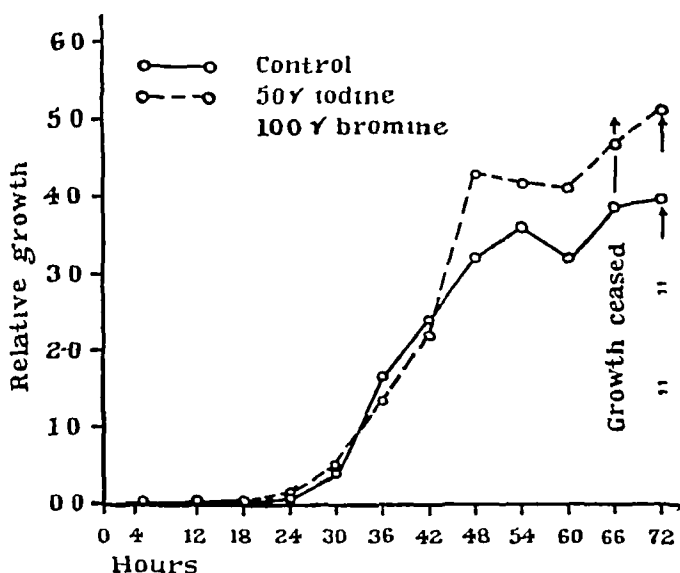
6 to 12 (controls) to which no iodine was added, and 6 to 12 to which iodine was added. The plasma used was of a different age in each experiment and, for this reason, the rate of growth in one experiment is not to be compared with that in another.

The results are shown in Text-figures 1 to 4 and in Plates X to XII, figs 5 to 10. They may be summarized as follows —

- (1) The addition of 12.5 γ of iodine per 100 c.c. of the culture-medium had little effect either on the rate of growth of the thyroid tissue *in vitro* or on the metabolism of the cells (Text-fig. 2). If anything, the effect of this concentration of iodine was slightly stimulating.
- (2) The addition of 25.0 γ of iodine per 100 c.c. of the culture-medium had a pronounced stimulating effect on the growth of thyroid tissue *in vitro* (Text-fig. 2). It also increased the rate of metabolism of the growing cells as judged by the rapidity with which liquefaction of the medium occurred (Plate X, figs 5 and 6).
- (3) The addition of 50.0 γ of iodine per 100 c.c. of the culture-medium increased the rate of metabolism of the growing cells but had only a slight stimulating effect on their rate of growth (Text-fig. 3 and Plate XI,



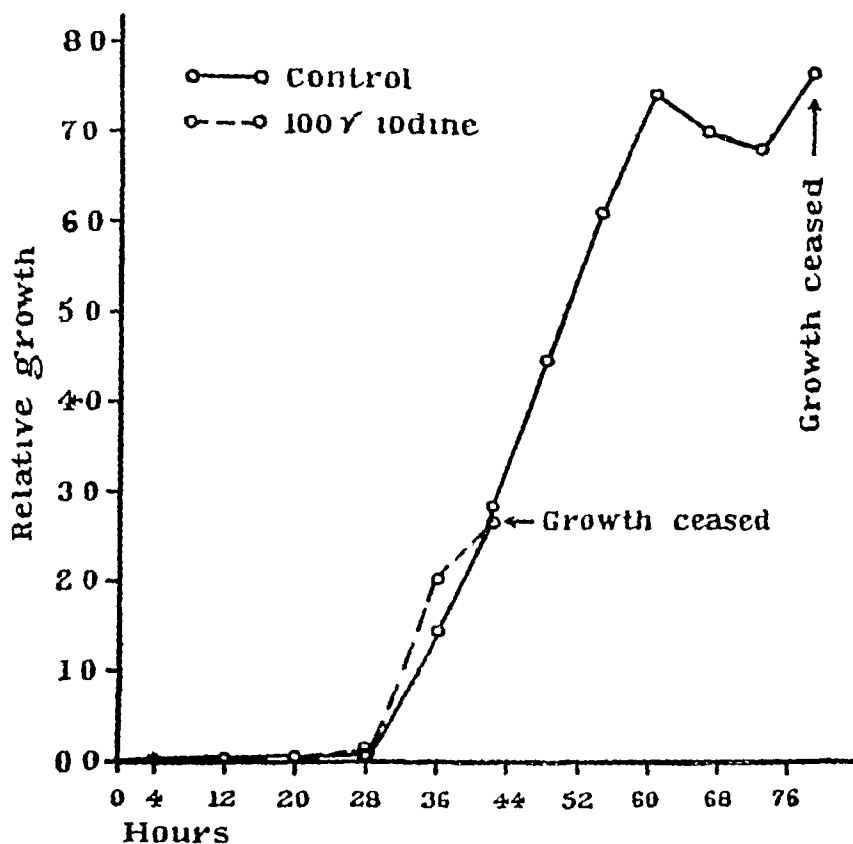
Text fig 2 Showing curve of growth of thyroid tissue *in vitro* as affected by the additions of 12.5γ and 250γ of iodine per 100 c.c. of the culture medium



Text fig 3 Showing curve of growth of thyroid tissue *in vitro* as affected by the addition of 500γ of iodine and of 1000γ of bromine per 100 c.c. of the culture medium

figs 7 and 8) In this experiment the effect of this concentration of iodine was compared with that of 100 0γ bromine It will be noted (Text-fig 3) that the latter had approximately the same effect as the former

- (4) The addition of 100 0γ of iodine per 100 c c of the culture-medium greatly enhanced the rate of metabolism of the growing cells and halved the duration of their life *in vitro* growth ceasing at the fortieth hour Up to this hour the rate of growth was the same as in the control series (Text-fig 4 and Plate XII, figs 9 and 10)



Text fig 4 Showing curve of growth of thyroid tissue *in vitro* as affected by the addition of 100 0γ of iodine per 100 c c of the culture medium

From these observations it would appear that iodine has different effects on growing thyroid tissue depending on its concentration in the plasma At a concentration of $X+25\ 0\gamma$, where X is the original iodine-content of the medium, it had a markedly stimulating action on their growth At higher concentrations ($X+50\ 0\gamma$ to $X+100\ 0\gamma$) it augmented the metabolism of the cells but shortened their life *in vitro* In the latter circumstance its action recalls the effects produced by its administration to tadpoles their growth is retarded while their metamorphosis is hastened

Recent observations, the results of which will be published at a later date, appear to indicate that these effects of iodine are not limited to thyroid tissue, but that similar effects on certain other tissues are produced by it

PLATE VIII

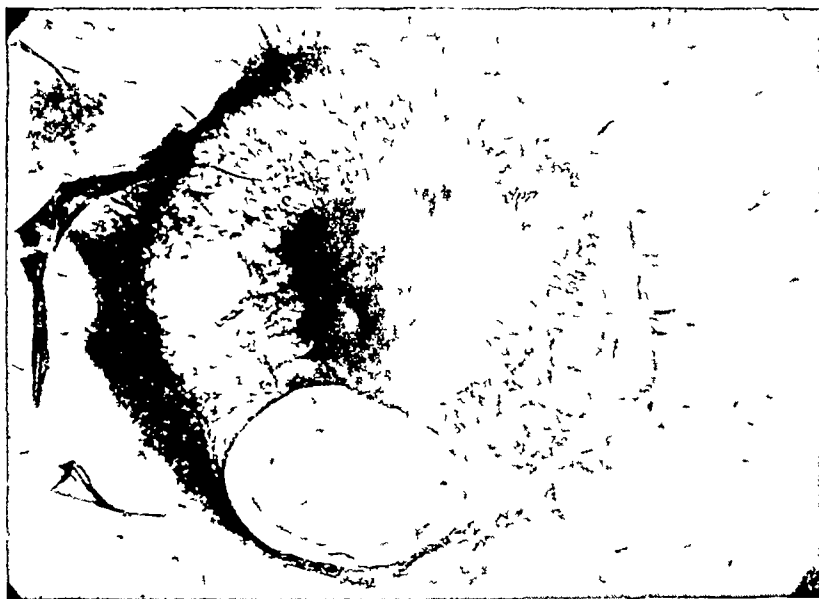


Fig 1 60 hour growth of thyroid Stained hæmatoxylin Note large vacuole at lower pole and another at left margin These are due to digestion and liquefaction of the plasma clot



Fig 2 80 hour growth of thyroid Stained hæmatoxylin Note formation of ring due to liquefaction of the plasma clot, and surface epithelial growth

PLATE IX.

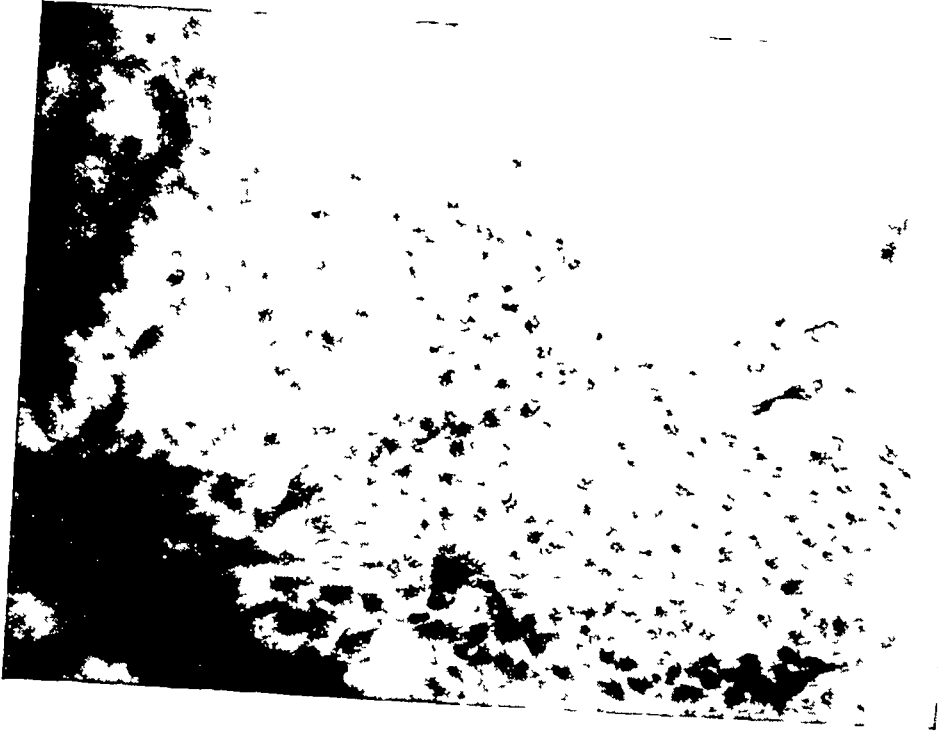


Fig 3 Thyroid epithelium growing *in vitro*.

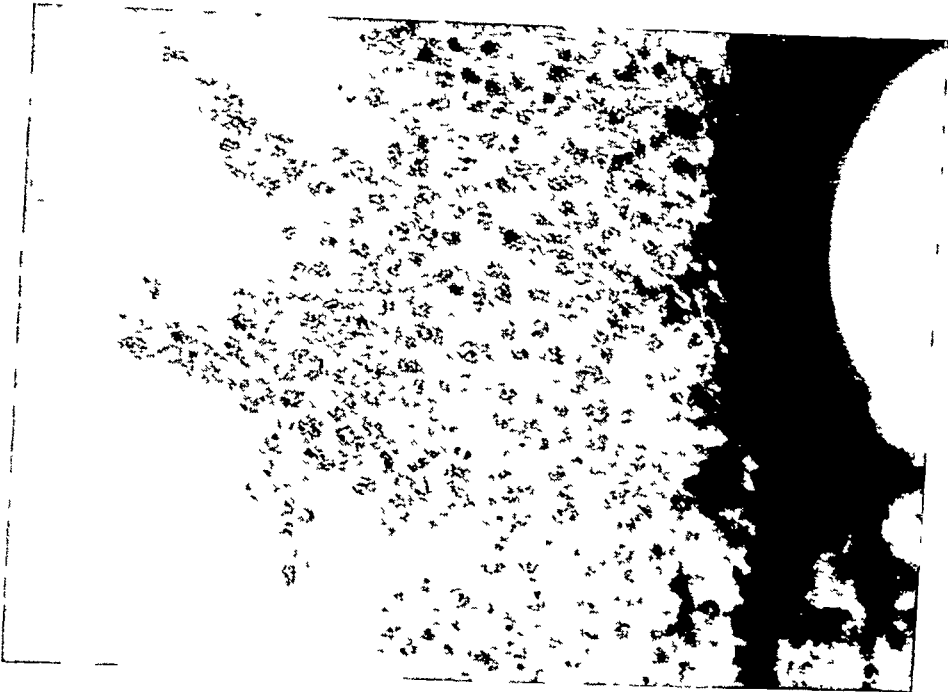


Fig 4 Thyroid epithelium growing *in vitro*.

PLATE X.

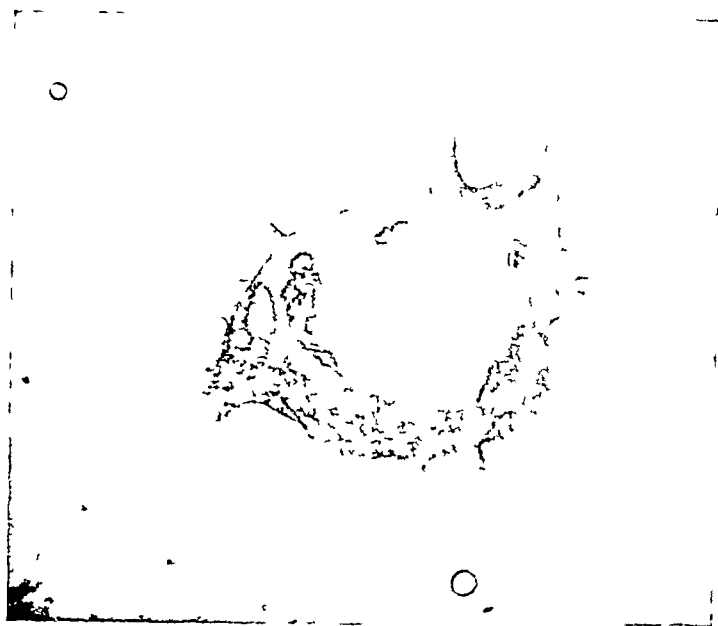


Fig 5 48 hour growth of thyroid tissue Stained hæmatoxylin
No iodine was added to the medium Plasma used was 15 days old
Compare with Fig 6

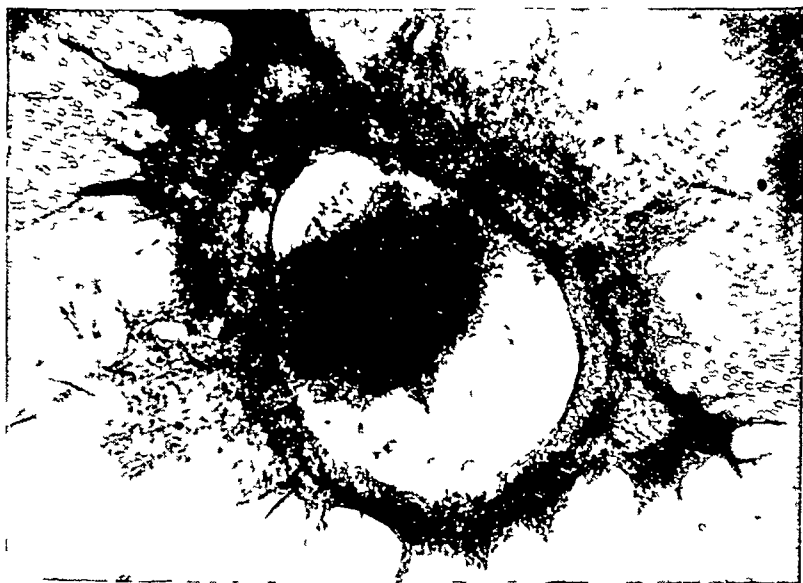


Fig 6 48 hour growth of thyroid tissue Stained hæmatoxylin 250 γ of
iodine was added to the medium Plasma used was 15 days old Note enhanced
growth and metabolism as compared with its control (Fig 5)

EFFECT OF PLASMA FROM A CASE OF *POLYNEURITIS*
GALLINARUM ON THE GROWTH OF TISSUES
IN VITRO

PRELIMINARY NOTE

BY

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Polyneuritis gallinarum was produced in a one year old black minorca hen by means of a diet composed of washed polished rice, autoclaved *dhal* (*Cajanus indicus*), cod-liver oil, and orange juice the three last ingredients being administered artificially. The main deficiency of this diet was one of vitamin B₁. The bird was bled at the onset of polyneuritic symptoms. The plasma obtained from it was of a greenish-yellow colour contrasting in this regard with the faintly yellow tinge of normal plasma from healthy control fowls. Its pH was normal.

The tissues used were (a) nerve-cells of the spinal cord of the 8 to 9-day chick-embryo, (b) the thyroid gland of the 18-day chick-embryo, and (c) fibroblasts of the heart of the 8 to 9-day chick-embryo.

These were explanted in a culture-medium consisting of plasma 50 parts and Tyrode solution 50 parts,—clotting of the medium occurring in the incubator. The cover-slip method was used.

Cultures were put up in both normal fowl-plasma (controls) and in the 'deficient' plasma,—the rate of growth in the one being compared with that in the other.

After 24 hours incubation growth of fibroblasts and nerve-cells had occurred both in the 'control' and 'deficient' plasma. There was the usual lag before growth of thyroid tissue began, after 48 hours incubation, growth of this tissue had occurred in both plasmas. But the various growths differed both qualitatively and

quantitatively in the two plasmas. In the deficient plasma the area of growth of *fibroblasts* was definitely smaller than in the control plasma, while the cells were long, thin and lanky as compared with the well-formed, fusiform and rounded cells which formed a thick matted growth in the control plasma.

Nerve-cells showed only scanty axis-cylinder outgrowths in the deficient plasma and no migration into the field from the explant. In the control plasma migration of nerve-cells into the surrounding medium was well marked, whilst growth was vigorous and presented the usual characteristics. It was observed that such scanty growth as did occur in the deficient plasma underwent rapid degeneration.

The differences presented by *thyroid tissue* in the two media were mainly quantitative, growth in the control medium being much more vigorous.

Sub-cultures of these tissues were made after 48 hours, the control cultures into normal plasma, and those in deficient plasma into deficient plasma. After 24 hours incubation the control sub-cultures were found to be growing vigorously while those in the deficient plasma showed little or no growth, not only so but such growth of nerve-cells as had occurred underwent rapid degeneration, the deficient plasma appeared to have a toxic effect on the nerve-cells.

Having failed to exhibit anything but very scanty growth in the first sub-culture a second sub-culture of thyroid tissue was made into normal plasma. After 24 hours incubation in this plasma the tissue was found to be growing well though not so well as when grown in control plasma from the first. A third sub-culture into fresh control plasma resulted in a further improvement in growth though the growth was still not so good as in controls.

It is apparent from these observations that the deficient plasma was not only lacking in a growth-promoting factor or factors (probably vitamin B₁) but that it caused degeneration of nerve-cells that had already grown. The effect of the deficient plasma on the growth of nerve-cells is shown in Plate XIII, figs 1 and 2.

PLATE XIII



Fig 1 48 hour growth of living nerve cells (unstained) growing in a mixture of normal fowl plasma and Tyrode solution. Note arborescent growth faintly seen at the periphery of the field, and vigorous growth as compared with Fig 2 which is of same magnification.



Fig 2 48 hour growth of living nerve cells (unstained) growing in a mixture of deficient plasma—from a polyneuritic fowl—and Tyrode solution. Note scanty growth; a few short axis cylinder fibrils are to be seen at the upper pole of the explant.

THE INFLUENCE OF HYDROGEN ION CONCENTRATION OF THE MEDIUM UPON THE GROWTH OF FIBROBLASTS *IN VITRO*

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[Received for publication, March 20, 1933]

AMONG the various factors of the media used for the growth of tissues *in vitro* hydrogen ion concentration has been recognized as an important one (Fischer, 1921). The present paper deals with the results of an investigation in which an attempt was made to learn the optimum range of pH for the growth of chicken fibroblasts *in vitro*, and the changes in pH brought about in the medium as a result of that growth.

Technique.

The method employed was essentially the same as that of Carrel —

A clot is made, at the bottom of a Carrel flask, with fowl-plasma and the Tyrode extract of an 8 to 9-day old chick-embryo, the proportions of these two constituents being 3 parts of the former to 1 part of the latter. Small fragments of heart-tissue from an 8-day embryo-chick are explanted into this clot and covered over with a fluid layer of embryo extract. The flask is hermetically sealed with a rubber-cap and incubated for several days at about 38.4°C. The growth of the tissue is examined periodically under a low power of the microscope and the area of growth charted on squared paper with the help of the camera lucida. The number of squares covered on the graph-paper gives the area of growth.

With a view to arriving at a suitable procedure for bringing about variations in the hydrogen ion concentration of the medium, a preliminary titration of embryo extract was made as follows. One cubic centimetre of embryo extract and one cubic centimetre of Sorensen's acid potassium phosphate and sodium hydroxide solution buffers in the biological range were added to each of several clean Pyrex

glass test-tubes, and the resulting pH determined with the glass-electrode The results of two such experiments are given below —

EXPERIMENT 1		EXPERIMENT 2	
Tyrode	8.57 pH	Tyrode	8.48 pH
Embryo extract	7.15 "	Embryo extract	7.40 "
pH of buffer added	Resulting pH	pH of buffer added	Resulting pH
Tyrode	8.00	Tyrode	8.14
7.00	7.00	7.00	7.43
7.20	7.21	7.20	7.61
7.40	7.39	7.40	7.77
7.60	7.56	7.60	7.83
7.80	7.66	7.80	7.93
8.00	7.77	8.00	8.06

It will be noted in the first place that the pH of the Tyrode solution, although the latter was of identical composition in both experiments, differed Similarly the pH of the embryo extract, although prepared in an identical way, differed significantly on the two occasions Further, the curves obtained (Fig 1) on

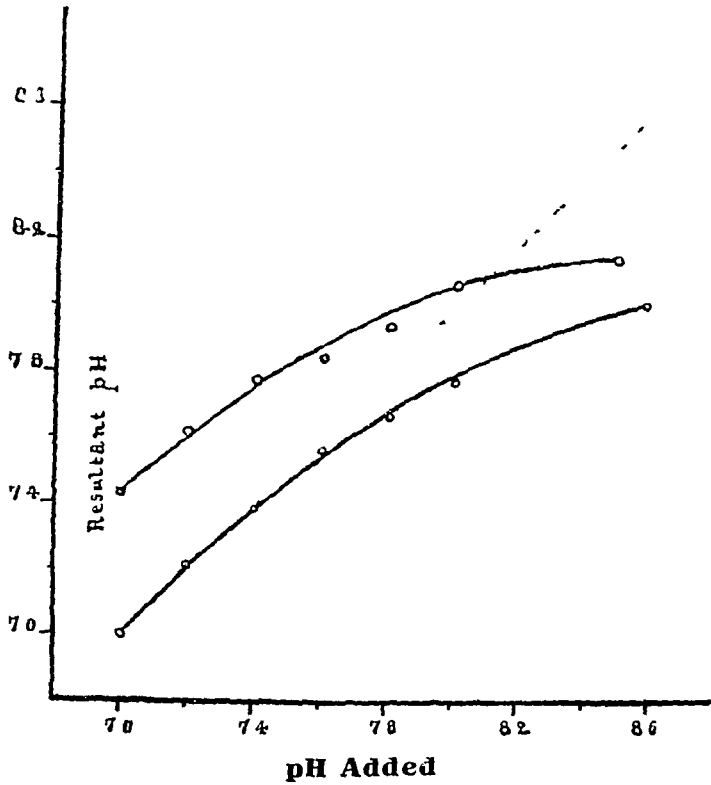


Fig 1 Showing the effect of adding buffers to the embryo extract

plotting out the results of the two experiments differed in slope and curvature according to the pH values of the Tyrode solution and embryo extract

Since hen-plasma forms part of the medium used in the actual cultural experiments it was considered necessary to take preliminary pH readings of the actual culture-medium. Accordingly, a clot was made by mixing hen-plasma and embryo-chick extract in each of several clean Curjel flasks, and a mixture of embryo extract and buffer was applied to the top of each clot. After about two hours, when equilibrium of the ions was established, the fluids were taken out with a capillary pipette for measurement of pH.

The following are the results of two such experiments (Fig. 2) —

EXPERIMENT 3

Clot 0.75 cc plasma and 0.25 cc embryo extract
Fluid medium 0.75 cc embryo extract and 0.75 cc buffer solution

Tyrosine	8.22 pH
Embryo extract	7.45 "
pH of the added buffer	Resulting pH
7.00	7.41
7.20	7.57
7.40	7.73
7.60	7.87
7.80	7.98
8.00	8.05

EXPERIMENT 4

Clot 0.5 cc plasma and 0.25 cc embryo extract
Fluid medium 1.0 cc embryo extract and 1.0 cc buffer solution

Tyrosine	8.51 pH
Embryo extract	7.21 "
Plasma	8.46 "
pH of the added buffer	Resulting pH
7.00	7.19
7.20	7.44
7.40	7.64
7.60	7.80
7.80	8.00
8.00	8.10

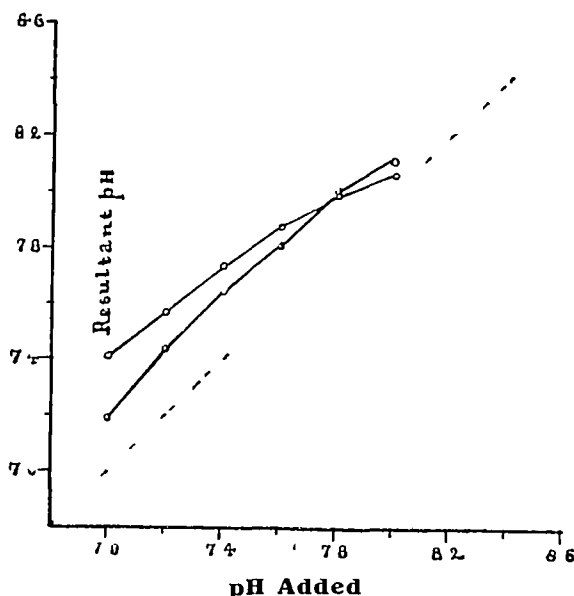


Fig. 2 Showing the effect of adding buffers to the plasma clot

glass test-tubes, and the resulting pH determined with the glass-electrode The results of two such experiments are given below —

EXPERIMENT 1		EXPERIMENT 2	
Tyrode	8.57 pH	Tyrode	8.48 pH
Embryo extract	7.15 "	Embryo extract	7.40 "
pH of buffer added	Resulting pH	pH of buffer added	Resulting pH
Tyrode	8.00	Tyrode	8.14
7.00	7.00	7.00	7.43
7.20	7.21	7.20	7.61
7.40	7.39	7.40	7.77
7.60	7.56	7.60	7.83
7.80	7.66	7.80	7.93
8.00	7.77	8.00	8.06

It will be noted in the first place that the pH of the Tyrode solution, although the latter was of identical composition in both experiments, differed Similarly the pH of the embryo extract, although prepared in an identical way, differed significantly on the two occasions Further, the curves obtained (Fig 1) on

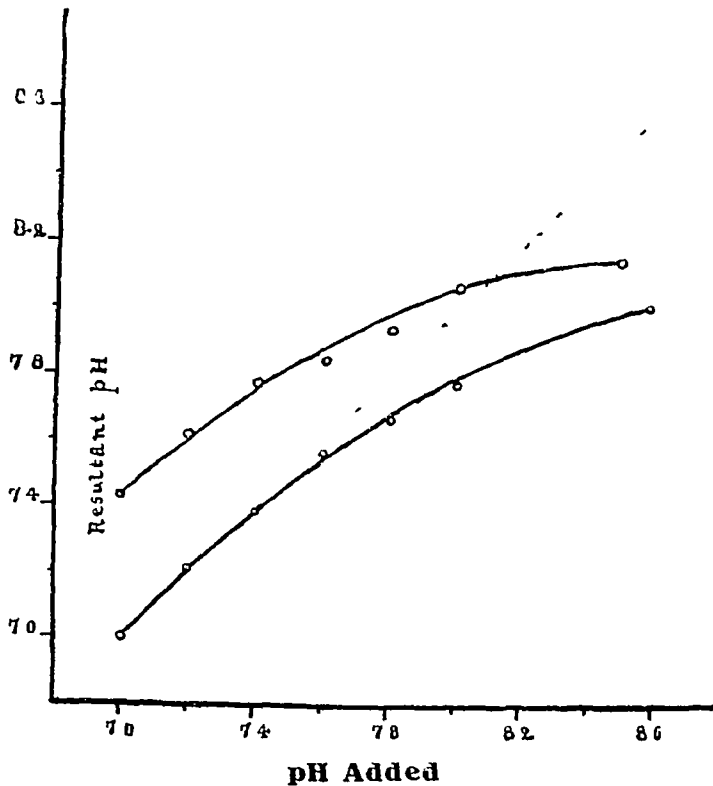


Fig 1 Showing the effect of adding buffers to the embryo extract

plotting out the results of the two experiments differed in slope and curvature according to the pH values of the Tyrode solution and embryo extract

these a fragment of chick-heart was explanted, the fragments being as far as possible of identical size. The explants were then incubated at about 38.4°C and the resultant growth of fibroblasts were measured every 12 to 24 hours in the way described above.

The results of one such experiment are tabulated below —

pH of medium	AREA OF GROWTH OF FIBROBLASTS IN DIFFERENT PERIODS			
	0 hours	12 hours	36 hours	60 hours
6.00	588	1,150	2,100	2,088
6.80	706	1,500	2,600	3,150
7.20	613	1,800	3,500	5,713
7.40	625	2,000	4,000	6,175
8.00	377	1,500	2,100	2,700

On plotting these figures on a graph (Fig. 4) it is seen that optimum growth occurs at about a pH of 7.2. At either side of this pH the growth of fibroblasts is less luxuriant.

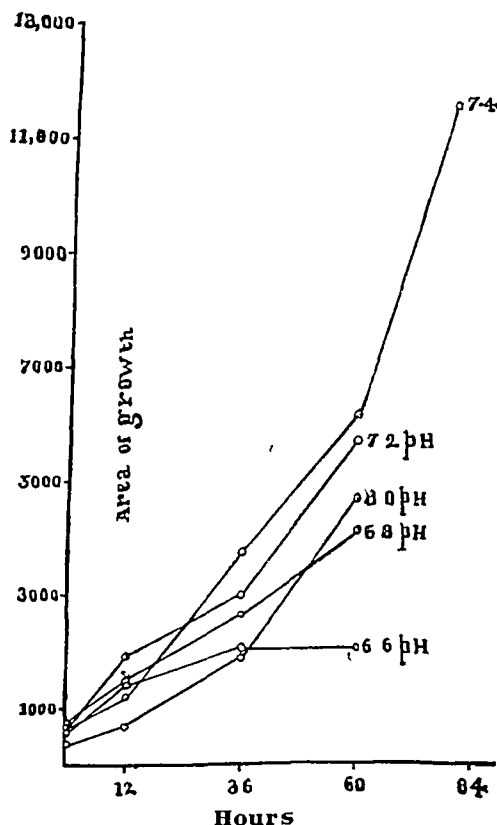


Fig. 4 Showing the growth curves of fibroblasts with different buffers added

Change in pH of medium resulting from growth of fibroblasts.

An attempt was then made to study the changes in pH of the medium that are brought about by the growth of tissues. Two identical sets of flasks were put up,—the one set with tissues explanted in them and the other without. Both were incubated at 38.4°C. After 60 hours' incubation the fluids were taken out with sterile pipettes and then pH measured with the glass-electrode,—fresh fluid being added to the flasks. A similar measurement was made 12 hours later. The results are given below and are represented graphically in Fig. 5 —

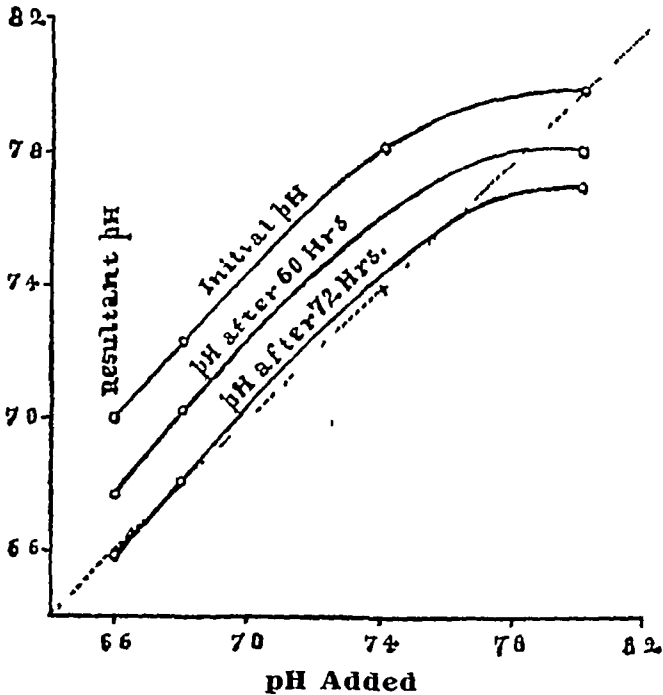


Fig. 5 Showing the shift in pH of the medium brought about by the growth of fibroblasts

pH of buffer	pH of medium without tissue	Resulting pH of medium after 60 hours' growth with tissue	After 72 hours' growth with tissue
6.60	7.00	6.77	6.58
6.80	7.23	7.02	6.81
7.40	7.82	7.40	7.20
8.00	8.00	7.82	7.71

As was to be expected the fluids became progressively more acid consequent, no doubt, on the metabolic activities of the growing tissue.

The areas of growth observed in this experiment were as under —

pH of buffer	pH of medium without tissue	AREA IN NUMBER OF SQUARES AT DIFFERENT PERIODS				Average pH of medium with tissue
		0 hours	18 hours	60 hours	72 hours	
6.60	7.00	800	840	2,195	2,195	6.88
6.80	7.23	450	4,800	7,100	7,300	7.18
7.40	7.82	450	4,800	8,800	12,000	7.61
8.00	8.00	230	1,185	1,710	1,710	7.91

Since there has been a progressive shift of pH with time due to growth of tissue it is not correct to say that the tissues were growing at a particular pH. It can, however, be said that the average of the initial and the pH after 60 hours can be

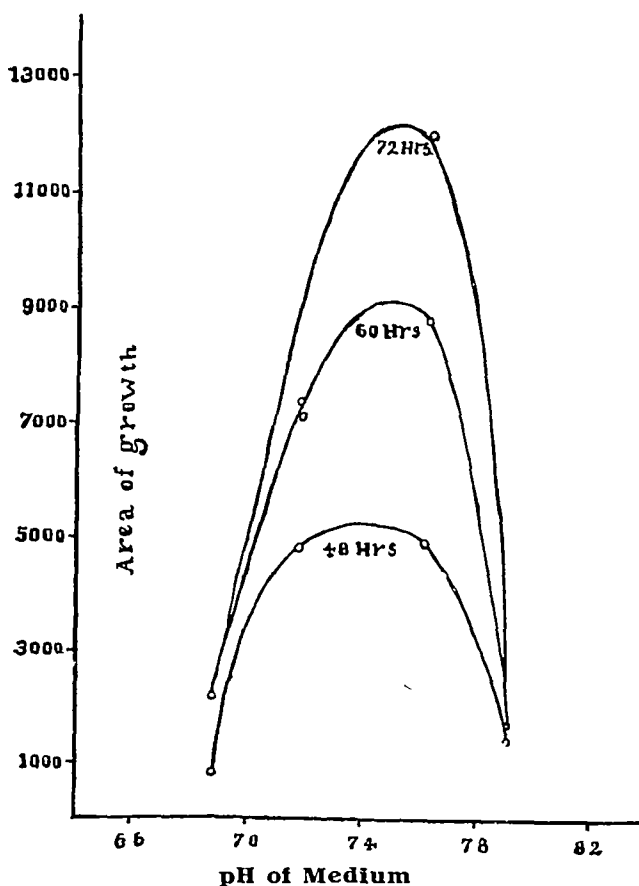


Fig 6 Showing the effect of pH of the medium on the growth of fibroblasts

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taken as the pH over a long period of time. Plotting such pH-value against area of growth at different periods of time typical inverted parabola (Fig. 6) is obtained which shows at a glance that growth is at a maximum between 7.4 and 7.6 pH and drops off on either side of this range of pH.

Another notable result of this experiment was that at a pH of 6.60 the clot turned cloudy after some time and that growth ceased after 60 hours. At 6.8 pH growth ceased after 72 hours. At 7.4 pH growth continued long after 72 hours, while at 8.0 pH growth was again inhibited at 60 hours.

Conclusions.

Fibroblasts of embryonic chick-heart when cultivated in the way described in this paper grew best at a limited range of pH,—namely 7.4 to 7.6.

Growth of fibroblasts is inhibited, after 60 hours, at ranges of pH both on the acid and alkaline side of 7.4 to 7.6.

Acknowledgments.

I wish to thank Professor Joseph Barcroft, F.R.S., Professor of Physiology, Cambridge, in whose department the work was done, Mr. A. L. Cochrane who worked with me, Mr. E. N. Willmer, Histologist to the Physiology School, for many helpful suggestions, and Dr. Honor B. Fell of Strangeways Laboratory for supply of plasma and other facilities.

REFERENCE

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CYANOGENESIS AND THIO-CYANOGENESIS IN FOOD-STUFFS¹

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Introduction

DURING the course of investigations on the nature of the goitrogenic agent in cabbage and in the 'noxa' associated with insanitary condition (McCarrison, 1933), evidence was obtained of the existence of cyanogen in them. This cyanogen was not present in the free state but in combination, probably as a cyanogenetic glucoside. The occurrence of cyanogenetic glucosides in plant materials is already known; dhurrin in *Sorghum vulgare*, gynocardin in *Gynocardia odorata* and *Pangium edule*, phaseolunatin in *Phaseolus lunatus*, *Linum usitatissimum* and in cassava, lotusin in *Lotus arabicus*, amygdalin in bitter almonds, and sinigrin in black mustard are some instances of cyanogenetic glucosides which have been isolated and studied. Apart from the interest that attaches to the isolation and determination of the chemical structure of the other cyanogenetic glucosides the object of the present investigation was to determine the amount of 'available' cyanogen in cabbage and in McCarrison's 'goitre-noxa', as also to examine other food-stuffs in common use in India from this standpoint.

Detection and estimation of cyanides in plant materials

The cyanogen being usually present in combination as a cyanogenetic glucoside, the first step in the process of its estimation is the splitting up of the glucoside. Various methods have been employed to this end—acid hydrolysis, maceration with water followed by steam distillation, and emulsin hydrolysis. Each method has its limitations, particularly the last two. Maceration with water is accompanied in most instances with a loss of cyanogen (Viehoefer, Johns, and Alsberg, 1916). Emulsin hydrolysis yields low results. Because of the well-known action of emulsin on amygdalin, prulaurasin (the cyanogenetic glucoside of common cherry laurel), and dhurrin, in liberating hydrocyanic acid, methods have been developed employing emulsin for the estimation of cyanides in plant materials. The fact that emulsin

* This work was carried out under the direction of Colonel Sir Robert McCarrison, to whom the author is indebted for many suggestions.—S R

does not react in the same way with such cyanogenetic glucosides as sinigrin, phaseolunatin, etc., seriously detracts from the universal application of the method. Acid hydrolysis appeared the least objectionable, though even here there were certain limitations to its adoption in general.

Distillation of the cabbage leaves in 5 per cent tartaric acid, 5 per cent hydrochloric acid and 3 per cent sulphuric acid were tried, as also autolysis of the material followed by steam distillation. The distillate was in every instance led into a solution containing a few drops of alkali. The yields of cyanogen were as follows —

	CN in mg per kilogram
Cabbage (5 per cent tartaric acid)	3.5
Cabbage (5 per cent hydrochloric acid)	3.2
Cabbage (3 per cent sulphuric acid)	6.3
Cabbage (autolysis)	0.2

It is seen from the above results that acid hydrolysis yielded better results than autolysis, and of the three acids employed, sulphuric acid in 3 per cent concentration was found to give the highest yield. Similar trials with other food-stuffs confirmed the above observation. Distillation in 3 per cent sulphuric acid was therefore adopted as the procedure in the estimation of cyanides recorded in this paper. The cyanogen thus liberated is termed the 'available' cyanogen. It does not necessarily represent the total amount of cyanogen that may be liberated by the specific enzymes associated with each cyanogenetic glucoside.

The accuracy of this procedure was again indicated by the good recovery of added cyanides. 0.1 mg., 0.2 mg., and 0.4 mg. of CN as sodium cyanide were added to weighed amounts of ground-nut (with husk) and the cyanogen-content estimated by distilling the sample in 3 per cent sulphuric acid. Allowing for the cyanogen that would be liberated from the ground-nut (with husk), the recoveries were 86.5, 90.8 and 94.8 per cent respectively.

The results of acid hydrolysis were in every instance checked against emulsin hydrolysis, the procedure adopted for the latter being that outlined by Roe (1924). Of the food-stuffs thus examined, emulsin hydrolysis gave, except in the case of linseed meal, a different reaction from that obtained by acid hydrolysis, details of which will be mentioned at a later stage.

Having thus obtained the best possible yield of cyanogen, the next step was the devising of a reliable method to estimate it. The methods in use for the micro-estimation of cyanides employ the production of the prussian blue colour and the matching of it with suitable standards treated likewise (Viehoever and Johns, 1915). Charlton (1926) has recommended the use of as much as 16 c.c. of 10 per cent ferrous sulphate solution, but the coloration due to this amount of iron salt was found to interfere with the estimation of cyanogen when the latter was present in small amounts—0.1 mg. and less in 100 c.c. Hence ferrous sulphate was used in much

smaller concentrations, ranging from 1 c c to 5 c c of a 10 per cent solution depending on the amount of cyanide present. Comparable standards were prepared. Where a precipitate of prussian blue was formed, aliquots were used so as to get only the greenish-blue colour.

The results yielded by the above method were checked against those obtained by another, based on the reduction of picric acid to isopurpuric acid by alkali cyanides. Guignard (1906) has employed this reaction for the detection of cyanogenetic glucosides by exposing test papers soaked in sodium picrate to the action of gaseous products of hydrolysis of the material under investigation for over 24 hours. This delicate but qualitative test was later refined and made fairly quantitative (Waller, 1910). Colour scales were employed and the change in colour of the test-paper brought about by its exposure to the material under examination was compared with standard ones exposed to known concentrations of cyanide solution, the period of exposure being constant in both. Better results were obtained by modifying Waller's method as follows. Aliquots of either the acid distillate or the solution obtained in Roe's method of emulsion hydrolysis of the food-stuff, absorbed in an alkaline solution, were just neutralized with dilute hydrochloric acid. The aliquots were so chosen as roughly to contain about 0.02 mg to 0.15 mg of CN. One c c, for every 50 c c of final volume, of a solution of sodium picrate rendered distinctly alkaline, was added and the solution made up to volume. The colour changes, on standing, to dark brown or yellowish-brown according as the cyanide is present in fair amounts or only in traces. When cyanide is not present, there is no change in colour even on standing for over a week. Standards (0.05 mg of CN in 50 c c is a convenient concentration while 0.2 mg, and over, produces too strong a colour for comparison) were set up for each set of estimations. The solutions were allowed to stand for about 72 hours, when colorimetric comparisons were made. The colour was found to increase up to 168 hours of standing after which it gradually faded, as will be seen from the accompanying results. Taking the colour produced by 0.05 mg of CN in 50 c c after standing for 72 hours as the standard, the colours produced at varying intervals of time were as follows —

Time interval	Coloration per cent
24 hours	41
48 "	76
72 "	100
96 "	104
120 "	111
144 "	118
168 "	121
192 "	103

Though the colour increased up to 168 hours, an interval of 72 hours was considered sufficient as solutions that were not visibly affected on standing for 48 to 72 hours were no more affected on standing for much longer periods. It is essential that in carrying out this method both the standard and the solution under examination should be allowed to stand for exactly the same time.

The results of cyanide estimation in food-stuffs by both these methods agreed fairly closely. There were one or two exceptions where the picrate method yielded higher results and in such instances the value obtained by the prussian blue method was taken as the more reliable. In other circumstances, the mean of the results of the two methods was taken as the final estimate. Commenting on Waller's method, Chapman (1910) points out that it should be used with caution as the colour produced is due to reduction and hence not specific for cyanogen. It is therefore likely that the higher yields obtained in a few instances may be due to the presence of other reducing substances. The results are incorporated in Table I.

As aforementioned the method of emulsin hydrolysis, as outlined by Roe, gave (except in the case of linseed meal) little or no cyanides in the food-stuffs examined. While emulsin reacts with the cyanogenetic glucoside present in linseed meal liberating free hydrocyanic acid as one of the end products of hydrolysis, it does not so react with the cyanogenetic glucosides present in the other food-stuffs examined. The failure to detect cyanides in the emulsin hydrolysate, or, if detected, to reveal its presence in amounts less than those obtained on acid hydrolysis, suggested the estimation of sulphocyanides in these hydrolysates. The emulsin hydrolysate gave, in most instances, strong tests for sulphocyanides whilst the distillates obtained on acid hydrolysis were, with the exception of mustard, free from them.

Two methods were employed for estimating sulphocyanides, the one serving as a control to the other—the ferric thiocyanate colorimetric method and the volumetric method of Sullivan and Dawson (1920). The sulphocyanide content given below represents the mean of the results obtained by the two methods —

TABLE I

Name of food stuff	ON 3 PER CENT SULPHURIC ACID HYDROLYSIS		ON EMULSIN HYDROLYSIS	
	Cyanide (as CN)	Sulphocyanide (as KCNS)	Cyanide (as CN)	Sulphocyanide (as KCNS)
Cabbage (fresh)	6.3 mg	<i>Nil</i>	0.8 mg	4.8 mg
Cabbage (96 hours old)			Trace	7.4 "
Polished rice	1.6 mg	<i>Nil</i>	Trace	21.7 "
Mustard	3.4 "	182.0 mg	3.8 mg	275.0 "
Milk (cow's)	2.9 "	<i>Nil</i>	Trace	34.1 "

TABLE I—*concl'd*

Name of food stuff	ON 3 PER CENT SULPHURIC ACID HYDROLYSIS		ON EMULSION HYDROLYSIS	
	Cyanide (as CN^-)	Sulphocyanide (as KCN^-)	Cyanide (as CN^-)	Sulphocyanide (as KCNS)
Oatmeal	2.1 mg	Nil	Nil	29.4 mg
Cholam (<i>Andropogon sorghum</i>)	4.3	Trace	1.5 mg	9.6 "
'Atta' (whole wheat flour)	2.0	Nil	Nil	35.4 "
Bran (wheat)	3.1	Nil	0.6 mg	87.6 "
Grass (green)	7.4	Nil	Nil	132.0 "
Soya bean	4.1	Nil	2.7 mg	Nil
Ground nut (with husk) (<i>Arachis hypogea</i>)	4.3 "	Nil	2.9 "	2.4 mg
Ground nut (without husk)	2.7 "	Nil	0.3 "	40.1 "
Ground nut husk	12.9	Nil	8.6 "	Nil
Sprouted gram (Bengal)	1.1 "	Nil	0.5 "	84.6 mg
Carrots	1.9 "	Nil	0.5 "	46.1 "
Mung dhal (<i>Phaseolus mungo</i>)	1.1 "	Nil	Trace	143.3 "
Dried yeast	4.3 "	Nil	1.3 mg	101.5 "
Vitamin A concentrate oil (B D H)	8.5 "	Nil	Trace	217.0 "
Linseed meal	358.5 "	Nil	648.0 mg *	Nil
Butter	1.8 "	Nil	1.6 "	Trace
Ragi (<i>Elysiue coracana</i>)	2.9 "	Nil	0.6 "	Trace
Lathyrus seeds	15.5 "	Nil	1.5 "	23.1 mg
Onion	11.3 "	Nil	2.0 "	Trace
Orange juice	3.5 "	Nil	0.6 "	Trace

[The above figures represent milligrams per kilo or per litre according as the material under examination is solid or liquid.]

* The figures obtained by titration against centinormal silver nitrate using potassium iodide as an internal indicator were slightly lower.

It is seen from Table I that cyanogen is present in varying amounts in the food-stuffs examined; that it is liberated in greater amounts by acid hydrolysis than by emulsion hydrolysis, the exception to this being linseed meal, and that

emulsin hydrolysis yields in most instances results which differ from those obtained on acid hydrolysis. Excluding linseed meal, it is to be noted that while acid splits up the cyanogenetic glucoside with the liberation of free hydrocyanic acid, emulsin splits it up into a sulphocyanide,—a reaction analogous to the action of emulsin on sinigrin. It appears as though the conversion of cyanogen to sulphocyanides by emulsin is, as compared with the liberation of hydrocyanic acid by acids, a process of detoxification of the cyanides. This process of detoxification also takes place in the gastro-intestinal tract of rats and rabbits consequent on enzyme action as is evident from Table II —

TABLE II

Showing results of hydrolysis of cabbage by enzymes present in

	Cyanide	Sulphocyanide
(1) Rabbit's stomach	0.6 mg	10.1 mg
(2) Upper and middle portion of the intestine of rabbit	0.5 "	15.2 "
(3) Lower portion of the intestine of rabbit	0.5 "	18.3 "
(4) Rats' stomach	Trace	26.4 "
(5) Upper and middle portion of the intestine of rats	Trace	36.0 "
(6) Lower portion of the intestine of rats	Trace	22.5 "

The figures represent milligrams per kilogram of cabbage

[In the above experiment the gastro-intestinal tract of one rabbit and that of three adult rats were used. The gastro-intestinal tracts were cut open, the contents removed and washed free from any adhering matter with distilled water. They were then ground in a mortar with glass powder and a few drops of glycerine, a little chloroform being added to prevent bacterial action. The finely ground mass freed from the glass powder was used for enzyme hydrolysis. The results reported above are all the more significant as the gastro-intestinal tract of one rabbit is, bulk for bulk, more than those of three rats.]

Reid Hunt (1905), and Reid Hunt and Seidell (1908) suggest that one of the possible means of detoxification of acetonitrile is through a combination of the CN molecule with a sulphur atom, whereby a relatively innocuous sulphocyanide is formed. They have also found that sulphur is an antidote for acetonitrile poisoning. The observations of Sullivan and Dawson are significant in this connexion. They find that (a) the production of sulphocyanide depends to a great extent on the nature of the protein and the amino-acid constituents thereof, and the capacity of the system to synthesize the sulphocyanide, (b) the differences in sulphocyanide content between individuals must be attributed to an increased assimilation and to an increase in detoxifying processes or the sulphocyanogenetic powers of the

organism as a whole, and (c) that the amount of sulphocyanide diminishes under conditions in which the activity of the nutritive function is diminished

The experiments with cabbage hydrolysed by the enzymes present in the gastro-intestinal tract of rats and rabbits as also the emulsin hydrolysis of the food-stuffs, do show that the cyanides are converted into sulphocyanides by enzymatic action. If this were a process of detoxification, as there is every reason to think, then the extent to which sulphocyanide rather than cyanide, is formed from a food-material containing a cyanogenetic glucoside on hydrolysis by either emulsin or the enzymes present in the gastro-intestinal tract, would serve as a measure of the toxic or non-toxic character of the food-material. Judged by this standard, whole milk, oatmeal, whole-wheat flour, green grass, sprouted gram, carrots, ground-nut (without husk), mung dhal, vitamin A-concentrate and polished rice would come into the category of non-toxic foods, mustard, cholam, cabbage, soya-bean, ground-nut (with husk), ground-nut husk, linseed meal, lathyrus seeds and onion in the category of toxic foods, while wheat bran, dried yeast, butter, ragi and orange juice will occupy an intermediate position between the two groups

Thio-cyanogenesis and anti-goitrogenic properties

McCarrison's researches on the nutritional aspect of goitre have enabled him to classify most of the food-stuffs examined here as possessing goitrogenic or anti-goitrogenic properties (McCarrison, 1931). He has found that while cabbage, bran, soya-bean and ground-nut possess distinct goitrogenic properties, carrots, sprouted gram and green grass (and whole milk) are anti-goitrogenic. The former belong, with the exception of bran, to the category of toxic food-stuffs, the latter to that of the non-toxic. It is, therefore, probable that an inverse relation exists between goitrogenic potency of a food-stuff and its thio-cyanogenetic power. The behaviour of wheat bran on emulsin hydrolysis is apparently irreconcilable with this hypothesis, whether there are any other factors concerned in making bran the exception in what appears to be a general rule cannot at present be said with certainty.

In addition to the above observation, viz., the existence of an inverse relation between goitrogenic index and thio-cyanogenetic power, the otherwise toxic nature of food-stuffs is reconcilable on the hypothesis that toxicity and cyanogenesis on enzyme hydrolysis run parallel, or, in other words, that non-toxicity and thio-cyanogenesis on enzyme hydrolysis run parallel. Thus the toxicity of linseed meal has been attributed to the cyanogen present in it (Almy and Robinson, 1920), cattle-poisoning in a Sorghum field immediately after a frost is traceable to the greater synthesis of cyanogenetic glucosides resistant to the detoxifying action of emulsin.

In conclusion, it may be said that the present paper represents only an attempt to indicate a possible relationship between cyanogenesis and thio-cyanogenesis in food-stuffs and their toxicity. Further work involving a greater number and variety of food-stuffs is necessary before definite conclusions can be formulated.

Summary and conclusions

(1) A number of food-stuffs were examined quantitatively for their cyanogen content, both by acid hydrolysis and by emulsin hydrolysis. Of those thus

practically no prophylactic value, the non-vaccinated controls having shown a mortality rate of 87 per cent when infected with the same test dose

As in the T A B vaccine manufactured at the Central Research Institute, Kasauli, the typhoid portion is represented by equal quantities of the Rawlings strain and of a locally isolated smooth strain (renewed at intervals), it was considered advisable to carry out a short investigation on the lines of Grinnell (*loc cit*) and determine whether or not the strains employed by us were of the desired protective value

The following points were studied —

- A Comparison of virulence of *B typhosus* (Rawlings) with that of a recently isolated smooth strain used at the present time in the preparation of anti-typhoid vaccine
- B Comparison of protection afforded to mice against an intraperitoneal infecting dose by the use of vaccines prepared from the Rawlings and freshly isolated smooth strains

PROCEDURE

EXPERIMENT 1

A Virulence

Mice weighing 20 to 25 grammes were inoculated intraperitoneally with 18 to 20 hours old Douglas broth cultures of *B typhosus* (Rawlings) and *B typhosus* (E D 402), a locally isolated smooth strain employed for vaccine production. Virulence results of these two strains are recorded below —

Strain of <i>B typhosus</i>	Number of animals	Dose in c c of 18 hours broth culture	Number of deaths	Percentage of mortality
Rawlings	20	0.15	20	100
402	20	0.15	20	100
Rawlings	20	0.1	10	50
402	20	0.1	16	80

It appears from the above that whereas an infecting dose of 0.15 c c causes 100 per cent mortality with both strains, a smaller dose of 0.1 c c shows the recently isolated, smooth strain No 402, to be more virulent than Rawlings

EXPERIMENT 2

B Active immunization of mice with killed broth cultures of
B typhosus

Two batches of 45 mice each were immunized with two doses of 0.1 c.c. of 18 hours old killed broth cultures of *B typhosus* (Rawlings) and *B typhosus* (E D 402) at 5 days' intervals. Ten days after the last immunizing dose a test dose of 0.15 c.c. of 18 hours broth culture of *B typhosus* (Karachi) was given intraperitoneally.

Percentage mortality and protection results are recorded below —

Number of vaccinated animals	Strain for vaccine	Strain for test dose	Survivals	Percentage of mortality during 7 days after the test dose	Percentage of survival rate
20	<i>B typhosus</i> (Rawlings)	<i>B typhosus</i> (Karachi)	20	0	100
20	<i>B typhosus</i> (E D 402)	<i>B typhosus</i> (Karachi)	20	0	100
25	<i>B typhosus</i> (E D 402)	<i>B typhosus</i> (E D 402)	25	0	100
22	<i>B typhosus</i> (Rawlings)	<i>B typhosus</i> (E D 402)	21	4.5	95.5
Controls	(non-vaccinated animals)				
20		<i>B typhosus</i> (Karachi)	5	75	25
20		<i>B typhosus</i> (E D 402)	4	80	20

Note — 1 Both these strains, viz., *B typhosus* (Rawlings) and *B typhosus* (E D 402) are employed for the manufacture of typhoid portion of T A B vaccine at Kasauli.

2 Broth cultures used for immunizing purposes were killed at 56°C for one hour and injected subcutaneously.

These results are in marked contrast with Grinnell's findings and indicate that the Rawlings strain used was of high protective value equal to that of the recently isolated smooth strain. The indications from this experiment were sufficiently definite to make it unnecessary to proceed further with tests on the same lines and it may be concluded that, provided the Rawlings strain is properly maintained and prepared by a suitable series of sub-cultures and its standard character confirmed before being used for the manufacture of vaccine, it will be satisfactory for the purpose. The fact that the Rawlings strain shows little tendency to dissociation

when properly kept makes it an extremely reliable organism for use in laboratories where fresh strains of standard characters are not easily obtainable, and, with other strains, whose characters have not been observed over a long period, the possible occurrence of variants may result in an unsatisfactory vaccine. For these reasons, and in view of the results now obtained, it would appear that the use of the standard Rawlings strain as a component of anti-typhoid vaccine is sound.

The value of vaccines for the prophylaxis of bacterial diseases which cannot be reproduced experimentally in animals has to a large extent been assessed on the results of agglutination and other serological tests but the results obtained in protection experiments are distinctly more convincing even when the disease is not typically reproduced. The method of intraperitoneal infection has been used to a considerable extent in connection with cholera work and from Grinnell's (*loc cit*) work and the experience in the present trials it would appear to be very suitable for use in tests of the typhoid-paratyphoid group. A trial was extended on the same lines to the stock strains of *B paratyphosus A* (Mears) and *B paratyphosus B* (Rowland) used as the other components of the T A B vaccine. In each case a preliminary test was made to determine the minimum dose producing cent per cent mortality on intraperitoneal injection of control white mice.

EXPERIMENT 3

C *Active immunization of mice with killed broth cultures of B paratyphosus A* (Mears)

A batch of 30 mice was immunized with two doses of 0.1 c.c. of 18 hours old killed broth culture of *B paratyphosus A* (Mears) at 5 days' intervals. Ten days later two minimum lethal doses* of live broth culture of *B paratyphosus A* (Felix) were given intraperitoneally.

Percentage mortality and protection results were as follows —

Number of vaccinated animals	Strain for vaccine	Strain for test dose	Number of MLD	Survival	Percentage of mortality	Percentage of survival rate
29	<i>B paratyphosus A</i> (Mears)	<i>B paratyphosus A</i> (Felix)	2	17	41.4	58.6
Control	(non-vaccinated animals)					
12		<i>B paratyphosus A</i> (Felix)	2	0	100	0

* Prior to the actual administration of test dose to vaccinated mice a preliminary test was made to determine the minimum dose of 18 hours broth culture which would cause cent per cent mortality on intraperitoneal injection of non-vaccinated mice. This dose was found to be 0.2 c.c. in the case of *B paratyphosus A* (Felix).

EXPERIMENT I

D Active immunization of mice with killed broth cultures of *B paratyphosus B* (Rowland)

A batch of 50 mice was vaccinated with two doses of 0.1 c.c. of 18 hours old killed broth culture of *B paratyphosus B* (Rowland) at 5 days' intervals. Ten days later 1½ and 2½ minimum lethal doses of *B paratyphosus B* (Tidy) were given intraperitoneally to separate groups.

Percentage mortality and protection results are recorded below —

Number of vaccinated animals	Strain for vaccine	Strain for test dose	Number of MLD	Survival	Percentage mortality	Percentage of survival rate
20	<i>B paratyphosus B</i> (Rowland)	<i>B paratyphosus B</i> (Tidy)	2½	7	65	35
Control		(non vaccinated)				
12		<i>B paratyphosus B</i> (Tidy)	2½	0	100	0
30	<i>B paratyphosus B</i> (Rowland)	<i>B paratyphosus B</i> (Tidy)	1½	25	16	84
Control		(non vaccinated)				
15		<i>B paratyphosus B</i> (Tidy)	1½	0	100	0

* Prior to the actual administration of test dose to vaccinated mice a preliminary test was made to determine the minimum dose of 18 hours broth culture which would cause cent per cent mortality on intraperitoneal injection of non vaccinated mice. This dose was found to be 0.15 c.c. in the case of *B paratyphosus B* (Tidy).

Note — Broth culture was killed by heating it to 56°C for one hour and injected subcutaneously.

It will be noted that while a high degree of protection was obtained against 1½ lethal doses in one of these tests an increase of dosage resulted in a marked reduction in the number of survivals. At a sufficiently high dosage no protection would probably be shown. The necessity for adjusting carefully the dosage of the infecting test material in order to obtain differential results is evident.

CONCLUSIONS

1. A very high degree of protection against intraperitoneal infection of mice was obtained by the use of a vaccine prepared from a smooth recently isolated strain of *B typhosus*.

Practically equal protection was obtained by the use of a vaccine of the 'fixed' type (Harvey, 1929) of the Rawlins strain.

Using exactly the same technique the results obtained with the Rawlings strain were in marked contrast with those of Grinnell who used Rawlings strain of smooth, intermediate and rough characters¹

The use of smooth recently isolated strains for vaccine purposes is sound but the maintenance of their character should be observed. The 'fixed' type of Rawlings strain is useful for the purpose and it may conveniently be used as one component of the vaccine.

2 The method of testing also applied to the stock strains *B paratyphosus A* (Mearns) and *B paratyphosus B* (Rouland) showed these strains to be of considerable protective value.

3 In testing by this method a careful adjustment of the infecting dose was necessary to obtain differential results.

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FURTHER OBSERVATIONS ON VITAMIN B₂

BY

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AND

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IN an earlier paper (Guha, 1931a), various materials, such as brewer's and baker's yeasts, milk powder, beef-muscle, ox-liver, etc., were investigated with reference to their potency in order to find the richest substance which might serve as a convenient starting material for the concentration and isolation of vitamin B₂. Of these materials, ox-liver was found to be the most potent source of the vitamin.

The present paper is the outcome of further investigations in this direction. Extracts of the livers of widely different species, like the buffalo, sheep, goat, pig, fowl and *Hilsa* fish (*Clupea Ilisha*, a common fish of Bengal) have been investigated with regard to their vitamin B₂ content. The potency of the extracts of ox-spleen, ox-muscle, and ox-kidney has also been studied.

EXPERIMENTAL

Biological technique — The technique for the assay of vitamin B₂ is essentially the same as has been previously described (Guha, 1931a). Young rats of about 50 g. in weight were fed on a basal diet of the following composition —

Starch (Cassava)	75 parts
Casein, 'Light White' (B D H)	21 „
Salt Mixture (McCollum's)	4 „

In addition each of the animals received daily 2 drops of a potent cod-liver oil to supply vitamins A and D. An extract of vitamin B₁ was prepared from rice-polishings in the following manner —

Rice-polishings (1 kg.) were extracted with 3 litres of boiling water, previously acidified with 6 c.c. of concentrated HCl for 6 minutes. After filtration under suction the filtrate was treated with saturated neutral lead acetate solution (about 100 c.c.) and left overnight. The precipitate was removed and the solution freed

from lead by hydrogen sulphide and subsequently from hydrogen sulphide by concentration *in vacuo*. This was fed in a daily dose corresponding to 2.5 g of rice-polishings. While practically free from B₂ it was a rich source of vitamin B₁ as shown by the biological tests. When the animals began to decline on this diet the extracts to be tested for vitamin B₂ were fed at different levels. In the absence of an international unit of vitamin B₂, a unit of vitamin B₂ was taken to be the amount which, administered daily to young vitamin B₂-deficient rats, would produce an increase in weight of approximately 10 g per week for a period lasting two to three weeks. Positive controls with an ox-liver extract as the source of vitamin B₂ and negative controls with no vitamin B₂ supplement were run all the time.

The general procedure of preparing the vitamin B₂ extracts was as follows —

The fresh tissue was finely minced and extracted with approximately 150 c.c. of boiling distilled water per 100 g of tissue. After boiling for 4 minutes the extracts were filtered under suction, the residues washed well and washings mixed with the main bulk of the extract.

Table I gives a summary of the results obtained (see also Figs. 1 and 2) —

TABLE I

Aqueous extract of material studied	Amount of tissue (in g.) equivalent to the day dose	Total solids per day-dose (mg.)	Average growth per week (g.)	Amount of tissue (in g.) containing one unit of vitamin B ₂	Units of vitamin B ₂ per 100 g. of tissue
Buffalo liver	1	72.2	+ 5.4	1.8	54
Sheep liver	0.8		+ 6	1.3	77
Goat liver	2	76.8	+ 8	2.25	44
Pig-liver	3	73.2	+ 6.5	4.6	22
Fowl-liver	1.8		+ 8	2.2	45
Hilsha fish liver	3		— 5		
Ox-liver (average)	2		+10	2	50
Ox liver (1 sample)	2		+21	1	100
Ox spleen	1.5		+ 9	1.6	63
Ox kidney	2.5	76.4	+16	1.5	67
Buffalo kidney*	1.6		+ 12.8	1.2	83
Ox-muscle	3.7		— 5		
Ox-muscle and ox-muscle residue	4		— 5		

* Extracted at pH 5

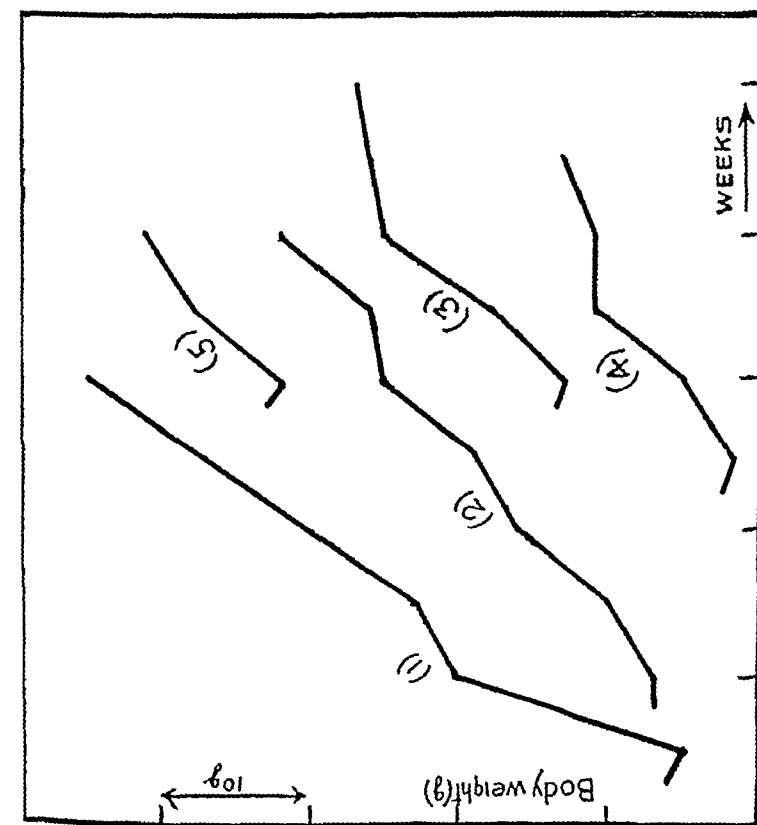


Fig 1

Curve (1) Ox kidney
 " (2) Pig liver
 " (3) Pig liver
 " (4) Sheep liver
 " (5) Ox spleen

Equivalent dose
 25 g
 18 g
 30 g
 08 g
 15 g

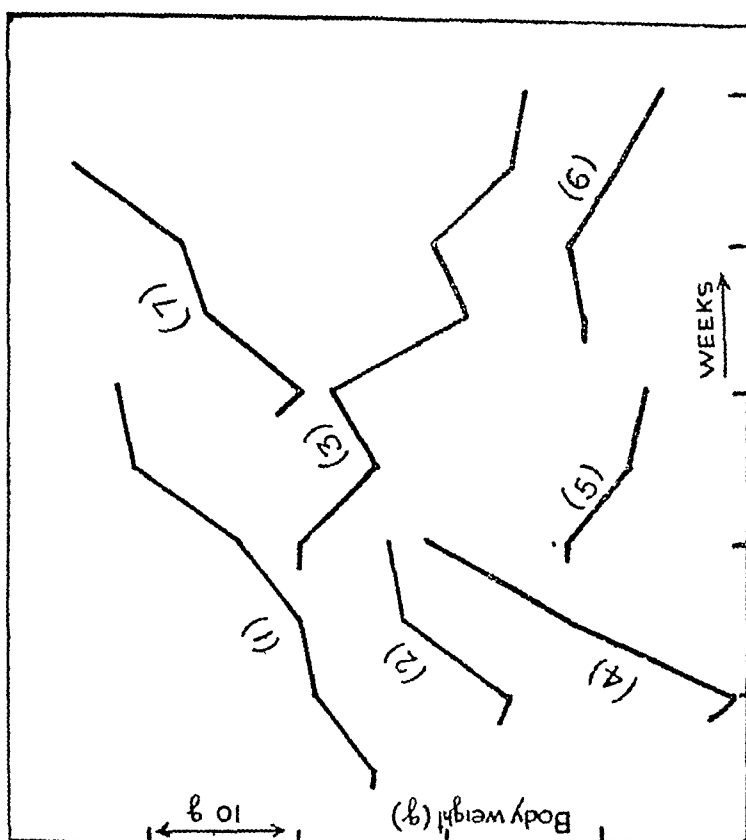


Fig 2

Curve (1) Buffalo liver
 " (2) Goat liver
 " (3) Halsia fish liver
 " (4) Ox liver (potent sample)
 " (5) Ox muscle
 " (6) Ox muscle (extract and residue)
 " (7) Ox liver (average)

Equivalent dose
 10 g
 20 g
 30 g
 20 g
 37 g
 40 g
 20 g

INFLUENCE OF pH ON THE EFFICIENCY OF EXTRACTION OF VITAMIN B₂

Some experiments were carried out to find the optimum pH for the extraction of vitamin B₂ from liver and kidney

A supply of buffalo-liver was finely minced and divided into three equal portions of 250 g. One portion of the above (250 g) was extracted for 4 minutes with boiling distilled water which was acidified with concentrated HCl to pH 1.6–1.8. This was filtered under suction and a yellow coloured-filtrate measuring 420 c.c. was obtained. Since the residue was slimy and coagulation was not complete, the filtration presented considerable difficulty [B. L. (4)]

Another portion (250 g) was extracted with the same amount of boiling distilled water for the same time, but this was previously brought to pH 3.5–3.7 by adding concentrated HCl. On filtering under suction a brown filtrate, measuring 575 c.c., was obtained [B. L. (5)]

The remaining portion (250 g) was extracted precisely in the same manner, in this case with acidulated water at pH 5.0. The yellowish-brown filtrate measured 575 c.c. [B. L. (6)]

Extraction of ox- and buffalo-kidney at pH 5.0—810 c.c. of water were acidified with concentrated HCl so that the pH value came to 5.0. 405 c.c. of this acidulated water were used for the extraction of 212 g. of ox-kidney in the usual manner [O. K. (3)]. The final volume of O. K. (3) was 450 c.c. The other portion of the acidified water was used for the extraction of 212 g. of buffalo-kidney [B. K. (3)]

Table II gives the vitamin B₂ values of the materials tested (see also Fig. 3)

TABLE II

Extract tested	pH of extraction	Average weekly growth (g)	Equivalent weight of liver fed per diem (g)	Units of vitamin B ₂ per 100 g. of tissue.
B. L. (4)	1.6–1.8	+ 3	3.8	8
B. L. (5)	3.5–3.7	+ 1.8	2.6	15
B. L. (6)	5.0	+ 12.2	2.6	50
O. K. (3)	5.0	+ 9	3	30
B. K. (3)	5.0	+ 12.8	1.6	83

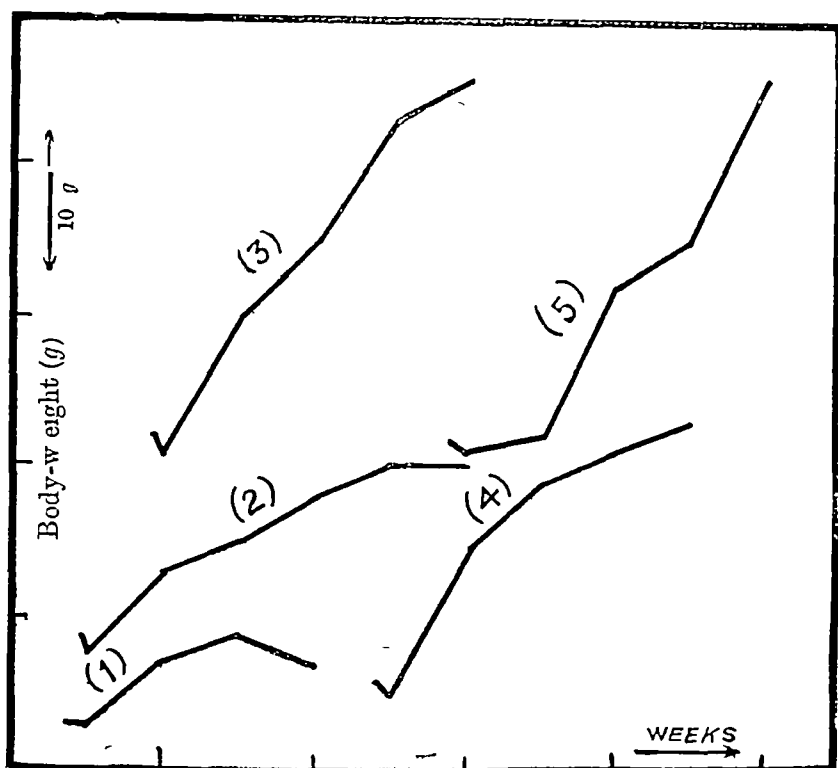


FIG 3

Curve (1) B L (4)
 „ (2) B L (5)
 „ (3) B L (6)
 „ (4) O K (3)
 „ (5) B K (5)

Equivalent dose
 38 g
 26 g
 26 g
 30 g
 16 g

STABILITY OF VITAMIN B₂

Reports regarding the stability of vitamin B₂ have in many cases been contradictory. While Goldberger *et al* (1926) showed that the anti-pellagra factor, which is now generally assumed to be identical with the growth-promoting vitamin B₂, was relatively much more heat-stable than vitamin B₁, Williams, Waterman, and Gurin (1929) and Chick and Roscoe (1930) have found that vitamin B₂ is not so heat-stable as it was thought to be. Thus, these workers found vitamin B₂ in yeast to be destroyed fairly effectively by autoclaving in an alkaline medium.

Guha (1931a) has recently shown that the stability of vitamin B₂ depends to a very large extent on the source from which the preparation has been obtained. Thus he found that dilute aqueous extracts of brewer's yeast and ox-liver deteriorate in vitamin B₂-potency on autoclaving for 3 hours at pH 9. But

vitamin B₂ in the commercial yeast extract 'Marmite', and the commercial liver concentrate of Eli Lilly's No 343, which has been found to be a potent source of vitamin B₂ (Guha, 1931b), was found to be relatively stable under the same conditions of treatment. Thus, the stability of vitamin B₂, like that of vitamin B₁ (Kinneisley and Peters, 1928, Guha and Drummond, 1929), appears to depend on associated materials.

Further experiments on the stability of vitamin B₂ are reported in this paper. It has been found that vitamin B₂ in ox-kidney extract is even more heat- and alkali-labile than that in ox-liver extract.

We attempted to ascertain whether certain materials, when added to liver extracts, could stabilize vitamin B₂ in them. Among the materials tried were saponin, gelatin, gum-arabic, egg-albumin and blood-albumin. The results, however, were disappointing. Table III summarizes the results obtained.

EXPERIMENTAL

1,250 c c of B L (2) (equivalent dose 946 g of fresh buffalo-liver) were treated with about 15 c c of 20 per cent NaOH, when the pH of the extract, which was originally at about 5, went up to about 9.0. The colour darkened on this treatment.

This extract was then divided into 5 fractions of 253 c c each. These were treated respectively with 25 c c of 1 per cent saponin (Merck) solution, 25 c c of 1 per cent gum-arabic solution, 25 c c of 1 per cent agar-agar (Merck) solution, 25 c c of 1 per cent gelatin (Merck's gold-label) solution and 25 c c of distilled water. They were kept in the alkaline state for 2 days in the refrigerator and were subsequently autoclaved for 3 hours under 1 atmosphere pressure. The pH of all the solutions changed to about 6. Each of them measured approximately 200 c c.

FURTHER PROTECTION EXPERIMENTS WITH GELATIN, EGG-ALBUMIN AND BLOOD-ALBUMIN

500 c c of B L (7) (equivalent dose 360 g of fresh buffalo-liver) were treated with about 55 c c of 20 per cent NaOH so that the pH of the extract went up to 9.0. The alkalinized extract was divided into two equal portions, to one of which were added 8 g of Merck's gold-label gelatin, the other portion serving as a control. Both of them were autoclaved simultaneously under 1 atmosphere pressure for 3 hours. The pH of the former changed to 6.5 while that of the latter fell to 6.8. Each solution was then made up to 250 c c by addition of distilled water.

In two other flasks, each containing 100 c c B L (7) (equivalent dose 72 g of fresh buffalo-liver), were added 5 g of egg-albumin and 5 g of blood-albumin (both Merck's) respectively. (Both were heated to 50°C to dissolve the albumins, but blood-albumin did not dissolve completely.) The pH of both of the preparations was then brought to 9.0 by adding 20 per cent NaOH. They were then autoclaved simultaneously for 3 hours under a pressure of 1 atmosphere. After autoclaving the pH of both the extracts came down to

7.2 The volume of each of the extracts was made up to 100 c.c. by adding distilled water

TABLE III

Protective used	Average growth per week (g)	Equivalent fresh liver fed per day dose (g)
Sapown	-13	5.4
Gum arabic	-5	5.4
Agar agar	-7	5.4
Gelatin	-3	5.4
Gelatin	-7	5.4
Egg albumin	-11	[5.6]
Blood albumin	-9	5.6
Negative control with distilled water	-5	5.4
Negative control with distilled water	-1	5.4
Positive control with untreated ox liver extract	+10	2
Positive control with untreated buffalo liver extract	+18	2

FURTHER EXPERIMENTS ON THE STABILITY OF VITAMIN B₂

4,550 c.c. of ox-liver extract (at pH 6.0-6.3) were brought to pH 10.8 with 20 per cent NaOH. This was then boiled for 5 minutes, made slightly acid with 27 c.c. of concentrated HCl and cooled. The final volume measured 4,150 c.c. [O. L. (29)]

500 c.c. of O. K. (11) (equivalent dose 214 g. of ox-kidney) were treated with 3 c.c. of 20 per cent NaOH, on which the pH value went above 10.5. The solution was refluxed on water-bath for 15 minutes and then made slightly acid with concentrated HCl. On alkalization and heating a precipitate, apparently of kidney protein, formed, which to a large extent remained undissolved even on acidification. The precipitate was not removed and the animals were fed with the mixture of the fluid and the precipitate [O. K. (12)]

AUTOCLAVING KIDNEY EXTRACTS FOR DIFFERENT PERIODS

Each of three lots of 200 c c of O K (4) (equivalent dose 105 g of ox-kidney) was treated with 13 c c of 20 per cent NaOH to bring the pH value to 9.0. These were autoclaved respectively for $\frac{1}{2}$ hour, 1 hour and 2 hours and called O K (8), O K (9) and O K (10) respectively. They were finally brought to pH 6.8 by the addition of HCl and tested.

TABLE IV
Showing the results of the experiments

	Average weekly growth (g)	Equivalent weight of fresh tissue fed per diem
O L (29)	-17	4 g
O K (12)	-4	2 g in the first week, subsequently raised to 2.5 g
O K (8)	+2.5	2.5 g in the first week, subsequently raised to 3.5 g
O K (9)	+1	2.5 g and subsequently 3.5 g
O K (10)	-6	2.5 g and subsequently 5 g

DISCUSSION

Units have recently been adopted for the measurement of vitamins A, D and B₁. There are now international standard preparations of vitamins D and B₁, to which preparations to be assayed are referred. For vitamin A the Carr and Price (1926) blue values are still extensively used, although recent evidence appears to point to the non-specificity of this colour reaction. In the absence of an international standard for the measurement of vitamin B₂, we have defined a unit of vitamin B₂ to be the amount, which on daily administration to young vitamin B₂-deficient rats, would produce an increase in weight of approximately 10 g per week for an experimental period of 2 to 3 weeks. We have found this standard to work fairly well.

The results reported in the present paper indicate that buffalo and ox-kidney extracts are the richest sources of vitamin B₂ among the materials studied. They have, however, been found to contain at the same time a little more vitamin B₁ than the liver extracts, which, as previously reported, are also potent sources of vitamin B₂. But although ox- and buffalo-livers are rich in vitamin B₂, it is a little surprising to find that their extracts are active only in equivalents of 2 to 2.5 g of fresh tissue. (Only one ox-liver sample was active in an equivalent of 1 g of tissue.) Ox-liver tissue, examined at Cambridge (Guha, 1931a), was found to be considerably more potent. This disparity may be due to racial differences.

and to the fact that animals slaughtered in England are usually much better fed than those killed in this country

Ox-muscle extract has been found to be singularly devoid of growth-promoting activity. This had also been observed earlier (Guha, 1931a). In the present investigation a combination of the muscle extract with the muscle residue was also fed with negative results. Beef-muscle has, on the other hand, been said to be a potent source of the anti-pellagra factor. These experiments again cast doubt on the prevalent theory that Goldberger's pellagra-preventive factor is identical with the factor called vitamin B₂, which supplements vitamin B₁ for the growth of young rats. Some suggestive observations bearing on this question have already been discussed (Guha, 1931c). There is reason to think that pellagra is a complex disease, of which the deficiency of vitamin B₂ is a contributory but probably not the sole cause.

Our experiments on the stability of vitamin B₂ support the earlier observation that the vitamin is not so stable as it was originally supposed to be. A considerable portion of the vitamin in ox-kidney extract is inactivated at pH 10.5 even by heating on the water-bath for 15 minutes. The distinction between vitamins B₁ and B₂ on the basis of a great difference in stability thus appears to be rather thin. Our attempts at stabilizing the vitamin by introducing foreign materials, like saponin, egg-albumin, etc., have so far proved futile.

SUMMARY

(1) A unit of vitamin B₂ has been defined to be the amount which, when administered daily to young vitamin B₂-deficient rats, would produce a weekly gain in weight of approximately 10 g for a period of 2 to 3 weeks.

(2) The amounts of vitamin B₂ in the tissues of various animals have been estimated in terms of vitamin B₂ units per 100 g of tissue. Ox- and buffalo-kidney extracts are the richest sources and contain 67 and 83 units of vitamin B₂ respectively per 100 g of tissue. Fowl-liver is a good source while *Hilsha* fish-liver appears to be a poor source of the vitamin.

(3) The extraction of vitamin B₂ from liver is optimum at pH 5.

(4) Ox-kidney extracts lose a large amount of vitamin B₂ on being heated on water-bath for 15 minutes at pH 10.5. Vitamin B₂ in ox-kidney extract therefore appears to be particularly labile. Efforts at the artificial stabilization of the vitamin have proved fruitless.

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NOTES ON SOME INDIAN SPECIES OF THE GENUS *PHLEBOTOMUS*

Part XXXIV

PHLEBOTOMUS IYENGARI N SP

BY

LIEUT-COLONEL J A SINTON, M D, D SC, I M S

(From the Malana Survey of India, Kasauli)

[Received for publication, April 5, 1933]

WHILE examining several collections of *Phlebotomus*, chiefly *P argentipes*, kindly sent by Mr M O T Iyengar from Travancore, two female specimens were found which were different from any of the other species of this insect described from India. Both these specimens were labelled 'Trivandrum, Travancore', and were collected in November and December 1932 respectively. It is proposed that this new species be named *Phlebotomus iyengari*.

Phlebotomus iyengari N SP (♀)

Appearances in dry state

Unfortunately no description of this can be given, because one specimen had been preserved in spirit and the other was not differentiated until it had been mounted in Canada balsam.

Appearances in stained and mounted specimens

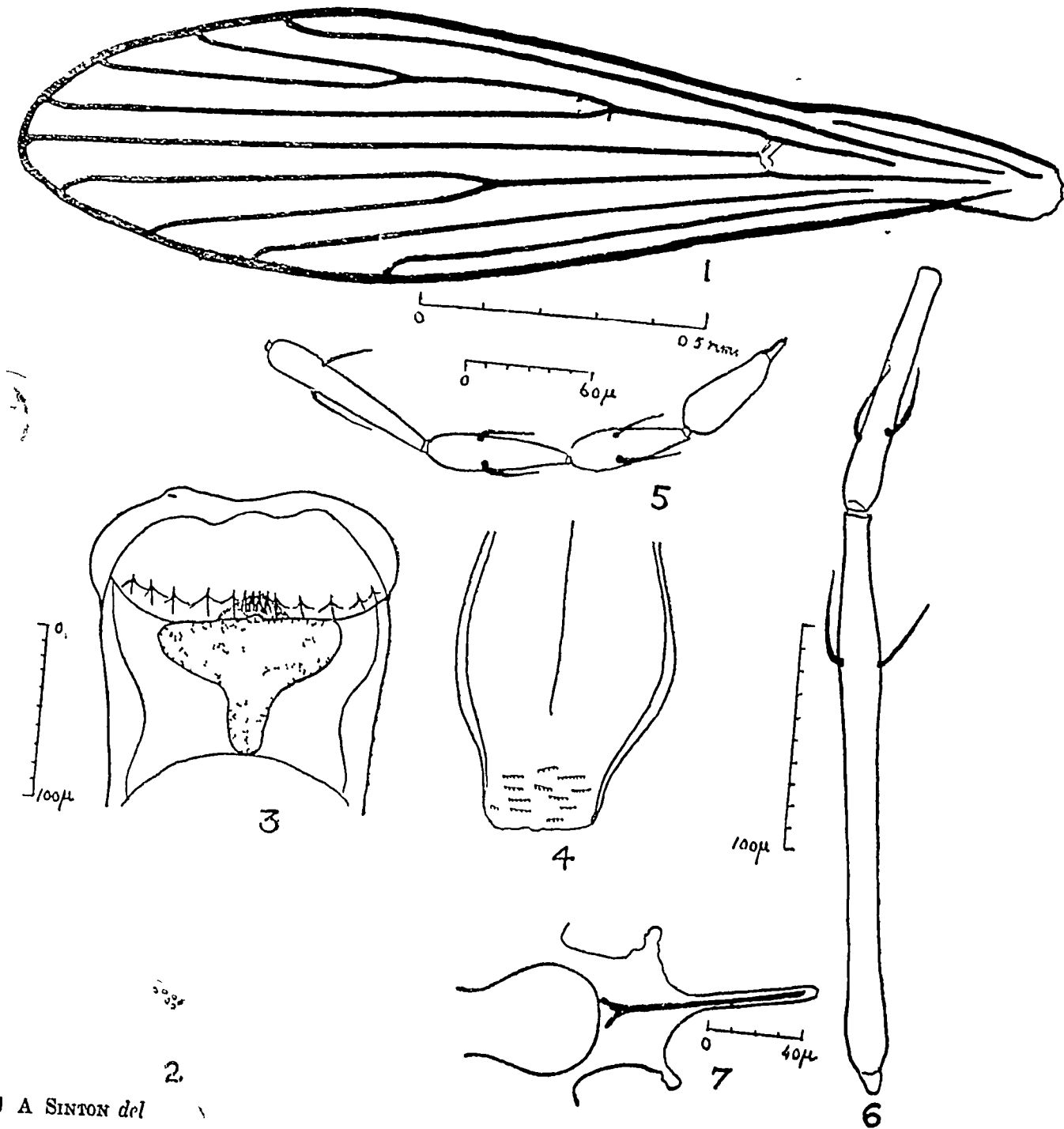
The measurements of the different parts of the two co-type females are given in the following Table.

The *total length* of the insect is about 2.5 mm. The clypeus is relatively very long. Scars of a few erect and many recumbent hairs are seen on the dorsal surfaces of abdominal segments II-VI.

TABLE

Phlebotomus iyengari N SP (♀)

Structures		Lengths in mm of specimens number —		Remarks, relative lengths, etc
		1	2	
BODY	Head and clypeus	0.400	0.400	
	Thorax	0.643	0.628	
	Abdomen proper	1.357	1.214	
	Superior clasper	0.185	0.170	
	Total length	2.60	2.40	
MOUTH	Labium		0.185	$\approx 3 \times$ breadth
	Pharynx, length		0.150	
	Pharynx, breadth		0.052	
ANTENNA	Segment III	0.255	0.200	III > IV+V
	Segment IV	0.108	0.093	
	Segment V	0.108	0.096	$\text{Formula} = \frac{2}{\text{III-XV}}$ $= 1.35 \times \text{IIIrd Seg}$ $= 6.0 \times \text{Seg III}, 4.4 \times \text{Segs XII-XVI}$
	Segment VI	0.110	0.100	
	Segments XII-XVI	0.345		
	Total length	1.530		
PALP	Segment 1	0.040	0.036	Formula—1, 2, 3, 4, 5 Rel lengths—2.7, 6.2, 8.9, 10, 19 $= 1+2$
	Segment 2	0.093	0.084	
	Segment 3		0.120	
	Segment 4		0.135	
	Segment 5		0.255	
	Total length		0.630	
WING	Length	1.914	1.800	$= 4 \times$ breadth
	Breadth	0.470	0.457	
	α	0.400	0.380	$\frac{\alpha}{\beta} = 1.12 - 1.32 \quad \frac{\beta}{\gamma} = 1.19 - 1.00$
	β	0.357	0.285	
	γ	0.300	0.285	$\frac{\delta}{\alpha} = 0.57 - 0.60 \quad \frac{\alpha}{\gamma} = 1.33 \quad \frac{\alpha}{\epsilon} = 0.73$
	δ	0.228	0.230	
	ϵ	0.543	0.514	$\frac{\theta}{\epsilon} = 1.77 - 1.83 \quad \frac{\alpha+\beta}{\theta} = 0.73 - 0.77$
	θ	0.985	0.914	
				$\frac{\text{Wing}}{\theta} = 1.94 - 1.97$



J A SINTON del

Phlebotomus iyengari n. sp. (♀)

Fig. 1. Wing. Fig. 2. Palp. Fig. 3. Buccal armature. Fig. 4. Pharyngeal armature. Fig. 5. Antenna, segments XIII-XVI. Fig. 6. Antenna, segments III-IV. Fig. 7. Furca.

TABLE—*concl'd*

Structures		Lengths in mm of specimens number —		Remarks, relative lengths, etc
		1	2	
HIND LEG	Femur	0.785	0.711	> 1 st length leg
	Tibia	1.043	0.957	> 1 st length leg
	Tarsus, seg 1	0.485	0.414	
	Tarsus, segs 2-5	0.685	0.600	
	Total length	3.00	2.70	(Not including coxa and trochanter)

The *buccal cavity* (Plate XIV, fig 3) has a well-developed pigmented area. The main portion of this is shaped like a segment of an orange with the convexity forwards. This convexity is continued forwards as a large blunt process, while from the middle of the straight posterior border arises a small, less highly chitinized, projection. The buccal armature has four small, narrow, central teeth and five large, broad ones on each side. These teeth are arranged in a row with the concavity backwards.

The *pharyngeal armature* (Plate XIV, fig 4) is very poorly developed.

The *antennæ* (Plate XIV, figs 5-6) have a formula of 2 over III to XV, the geniculate spines are slender and not very long. Segment III forms about $\frac{1}{8}$ th of the total appendage, it is greater than the combined lengths of segments IV and V, and equals about 1-7th of segments XII to XVI together. The distal end of segment III reaches to the end of the proboscis.

The *palps* (Plate XIV, fig 2) have a formula of 1, 2, 3, 4, 5, in anatomical order the relative lengths of the segments are about 2.7, 6.2, 8.9, 10, 19. Segments 1 to 4 are fairly stout, while segment 5 is long and thin. Newstead's spines are situated on the basal third of segment 3, and number about 15-20.

The *wing* (Plate XIV, fig 1) is lanceolate and about 4 times as long as broad. The ratio δ over α is about 0.6, α is very long and the ratio α over β is about 1.2. The fork of the 4th vein is placed very distally in the wing, being midway between the two forks of the 2nd vein.

The *spermathecae* were not visible.

Differential diagnosis

The occurrence of scanty erect hairs on the dorsal surfaces of abdominal segments II-VI differentiates this species from all the members of the recumbent-haired group.

The morphology of the buccal armature and pigmented area are distinctly different from any other species recorded in this group (sub-genus *Sintonius*,

Nitzulescu), except *P. tibnads* (Adler and Theodor, 1929, Adler, Theodor, and Lourie, 1930). In the latter species the palpal formula is 1, 2, 4, 3, 5, the total length of the insect is less than 2 mm, δ is very small and the ratio α over β is about 0.6.

The buccal armature in *P. vyengari* shows some resemblance to that of the recumbent-haired species, *P. dentatus* from Baluchistan (Sinton, 1933). The absence of a pigmented area in the latter species, the very curved alignment of the buccal armature, as well as the number and arrangement of the teeth, differentiate the two species. In addition *P. vyengari* has a very poorly developed pharyngeal armature and a different palpal formula.

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NOTES ON SOME INDIAN SPECIES OF THE GENUS *PHLEBOTOMUS*

Part XXXV.

ADDITIONS AND ALTERATIONS TO THE DIAGNOSTIC TABLE OF FEMALES

BY

LIEUT-COLONEL J. A. SINTON, M.D., D.Sc., I.M.S.

(From the Malaria Survey of India, Kasauli)

[Received for publication, April 5, 1933]

SINCE the diagnostic table for the females of the Indian species of the genus *Phlebotomus* was published about one year ago (Sinton, 1932a), several other species have been added to the list of Indian fauna. These include—

- (i) *P. stantoni*, an erect-haired species, of which one specimen was discovered in a collection made by Captain P. J. Barraud, F.E.S., F.Z.S., at Golaghat, Assam, in 1924*,
- (ii) Three new species belonging to the group with scanty erect hairs (sub-genus *Sintonius*)—(a) *P. eadithæ* from Saugor, Central Provinces (Sinton, 1932b), (b) *P. hodgsoni* from the North-West Frontier Province (Sinton, 1933b) and (c) *P. vyngari* from Travancore (Sinton, 1933c), and
- (iii) *P. dentatus*, a recumbent-haired species, from Quetta, Baluchistan (Sinton, 1933a)

In order that these five species should be included in the diagnostic table mentioned above, the following alterations and additions are given to replace items 8 to 13 of that table —

ADDITIONS AND ALTERATIONS IN TABLE FOR THE IDENTIFICATION OF THE FEMALES OF THE INDIAN SPECIES OF THE GENUS *PHLEBOTOMUS*

- 8 Spermatheca fusiform with striations forming triangular areas at margins (Plate III, fig 13)†, pharynx large and markedly flask shaped with armature consisting of large teeth about middle line and smaller teeth laterally (Plate III, fig 14)†

P. chinensis.

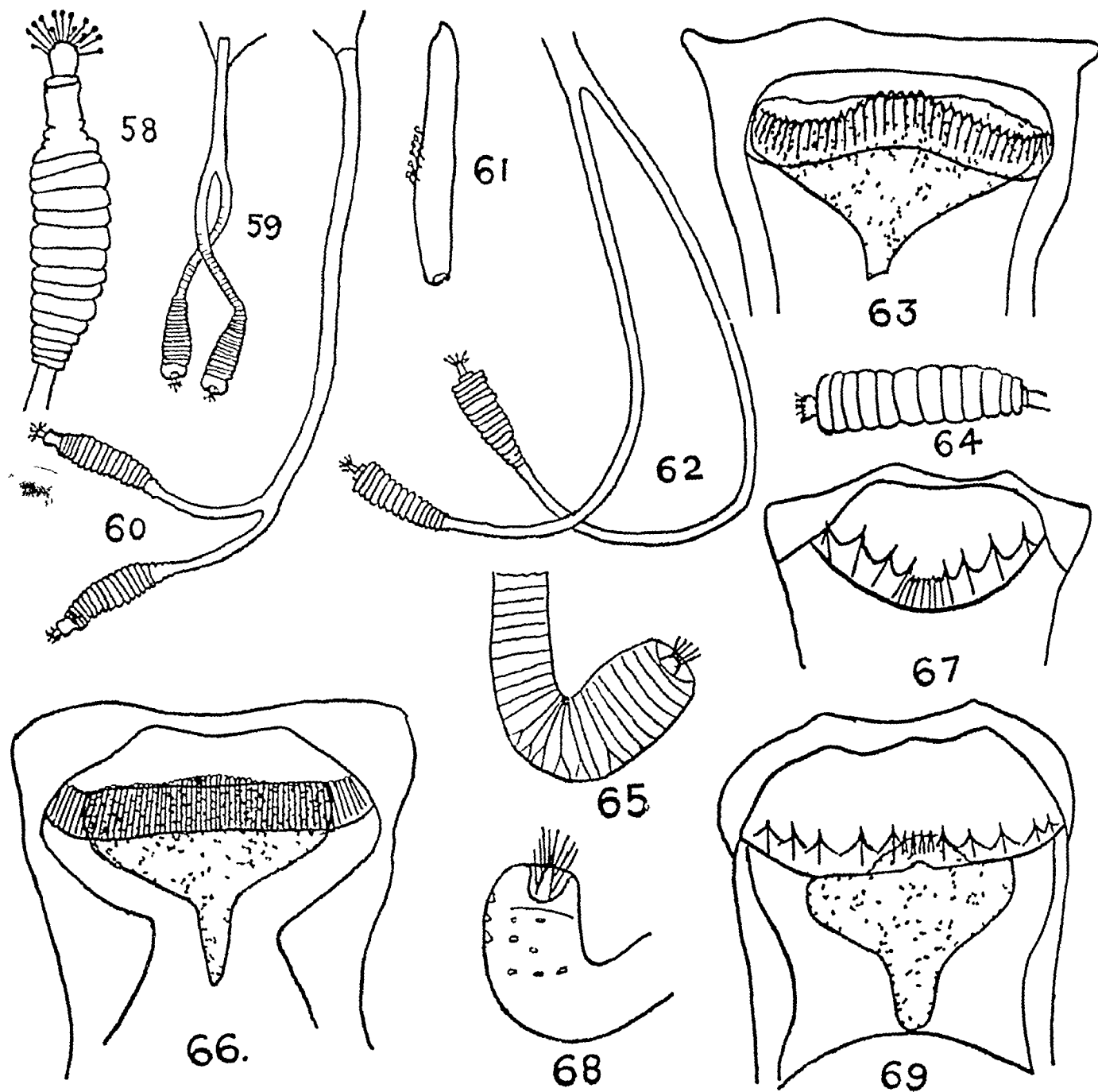
* A full description of the female of this species from Siam has been given by Sinton (1931)

† These numbers refer to the Plates issued with the original diagnostic table (Sinton, 1932a)

- Spermatheca with transverse striations (Plate III, figs 16 and 18)*, pharynx not markedly flask shaped, with armature showing small teeth about middle line and few or none laterally (Plate III, fig 15)* 8a
- 8a Spermatheca fusiform with small apical segment (Plate XV, fig 58), spermathecal ducts short but common duct very long (Plate XV, fig 60), geniculate spines long, Newstead's spines grouped around middle of palpal segment 3 (Plate XV, fig 61) **P. stantoni.**
- Spermatheca carrot shaped (Plate III, figs 16, 18)*, common spermathecal duct short (Plate XV, figs 59-62) 9
- 9 Spermatheca very markedly carrot shaped, with large apical segment (Plate III, fig 16)*, short spermathecal ducts, each not more than twice the length of body of organ (Plate XV, fig 59), sternal tubercle narrow (Plate III, fig 17)*, geniculate spines comparatively short, Newstead's spines scattered over middle third of 3rd palpal segment (Plate III, fig 16a)* **P. argentipes.**
- Spermatheca not so markedly carrot shaped, with small apical segment (Plate III, fig 18)*, long spermathecal ducts each about 4 times the length of the body of the organ (Plate XV, fig 62), sternal tubercle broad (Plate III, fig 19)*, geniculate spines long, Newstead's spines grouped near middle of 3rd palpal segment (Plate III, fig 17a)* **P. colabaensis.**
- 10 Spermatheca turnip shaped and unsegmented (Plate IV, fig 25)*, well developed buccal and pharyngeal armatures (Plate IV, figs 24 and 26)*, groups of wide scales on thoracic pleura, antennal formula 1 over IV-XV, Newstead's spines present on both 2nd and 3rd palpal segments (*vide* No 19) **P. squamipleuris.**
- Spermatheca elongated with distinct transverse segmentation (Plate III, fig 21)* (Plate XV, fig 65), antennal formula 2 over at least IV-XV, Newstead's spines on 3rd palpal segment only 11
- 11 Spermatheca pipe shaped with very wide duct (Plate XV, fig 65), well developed pigmented area and buccal armature with about 60 teeth (Plate XV, fig 66), palpal formula 1, 2, 4, 3, 5 **P. hodgsoni.**
- Spermatheca sausage shaped with 8-12 segments and narrow duct (Plate III, fig 21)* 11a
- 11a Pigmented area poorly developed and buccal armature with 4-6 widely separated teeth (Plate III, fig 20)*, palpal formula 1, 2, 4, 3, 5 **P. christophersi.**
- Pigmented area well developed and buccal armature with 10-60 contiguous teeth (Plate III, fig 23, and Plate IV, fig 28)* 12
- 12 Buccal armature with 10-15 teeth 12a
Buccal armature with more than 30 teeth 12b
- 12a Buccal teeth of about equal size, arranged in almost straight line (Plate III, fig 23)*, pigmented area with sharply pointed tail and almost straight posterior margin, antennal segment III less than IV and V together, palpal formula 1, 2, 4, 3, 5 **P. clydei.**

* These numbers refer to the Plates issued with the original diagnostic table (Sinton, 1932a)

PLATE XV.



J A SINTON del

Fig 58 Spermatheca of *P. stantoni* Fig 59 Spermathecae and ducts of *P. argentipes* Fig 60 Spermatheca and ducts of *P. stantoni* Fig 61 Third palpal segment of *P. stantoni* Fig 62 Spermathecae and ducts of *P. colabensis* Fig 63 Buccal cavity of *P. eadithae* Fig 64 Spermatheca of *P. eadithae* Fig 65 Spermatheca of *P. hodgsoni* Fig 66 Buccal cavity of *P. hodgsoni* Fig 67 Buccal cavity of *P. dentatus* Fig 68 Spermatheca of *P. dentatus* Fig 69 Buccal cavity of *P. yengari*

Buccal teeth very large laterally and small centrally arranged with distinct posterior concavity (Plate XV fig 69), pigmented area with blunt tail and central posterior projection, antennal segment III greater than IV and V together, palpal formula 1, 2, 3, 4, 5

P. iyengari.

- 12^b Buccal armature with about 35 teeth and pigmented area with slightly truncated tail (Plate XV, fig 63) spermatheca with 12 segments (Plate XV fig 64) palpal formula 1, 2, 3, 4, 5, antennal segment III less than IV and V together

P. eadithae.

Buccal armature with about 50-60 teeth and pigmented area with markedly truncated tail (Plate IV fig 28)* spermatheca with 8-10 segments palpal formula 1, 2, 3, 4, 5, antennal segment III greater than IV and V together

P. hospitil.

- 13 Pigmented area poorly developed or absent (Plate IV figs 30 and 35)*

13a

Pigmented area well developed (Plate IV figs 32, 33, 34, etc)*

16

- 13a Pharyngeal armature well developed and buccal armature with 6 smaller median teeth and 4 very large pointed teeth on each side (Plate XV fig 67) spermatheca large with wide duct (Plate XV fig 68), palpal formula 1, 2, 4, 3, 5

P. dentatus.

Pharyngeal armature poorly developed (Plate IV, figs 30 and 35)*

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NOTE OF A RAPID METHOD OF PREPARING SANDFLIES FOR EXAMINATION

In a previous paper (Sinton 1932a) lacto-phenol was advocated as a clearing agent for both fresh and dry specimens of *Phlebotomus*. Recent work with chloral-lacto-phenol, as described by Langeron (1921), has shown this fluid to be even better than lacto-phenol.

The composition of the fluid used is as follows —

Chloral hydrate (crystals)	2 parts
Acid, carbolic (crystals)	1 part
Lactic acid, pure	1 part

This fluid not only clears specimens rapidly but also quickly softens dried ones and makes them easy to dissect. Specimens cleared in this fluid show very distinctly the details of the buccal and pharyngeal armatures, as well as of the spermathecae.

The fluid has also been found very useful in examining the appendages of mosquitoes and other insects. The portion to be studied is placed in the fluid for $\frac{1}{2}$ -1 hour, or longer if necessary. It is then dissected, arranged and examined under a coverglass. If it is desired to make a permanent unstained preparation, the chloro-lacto-phenol is washed out with amyl alcohol, which is in turn replaced by xylol†.

* These numbers refer to the Plates issued with the original diagnostic table (Sinton, 1932a)

† This step may often be omitted, but the results are usually better and more certain if xylol is used

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and Canada balsam applied The method is easy, rapid and gives very good results

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Postscript —

While this paper was in the Press Dr Perfiljev has kindly sent me a reprint of a paper on the sandflies of Turkemenistan [*Zool Anzeiger*, (1930), **101**, 7/8, pp 221-27] In this paper he describes a new variety of *Phlebotomus*, *P. minutus* var *arpaklensis* This variety seems identical with *P. dentatus* but shows an oval pigmented area I have recently received a specimen answering to this description from Lieut Colonel H E Shortt, I M S This was collected between Malakand and Dir, N-W F Province —[J A S]

CONCENTRATION OF ANTIVENOMOUS SERUM

BY

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[Received for publication, April 12, 1933]

IN a previous communication (Mallick and Maitra, 1932), certain preliminary work bearing on concentration of the antivenomous serum prepared at the Central Research Institute, Kasauli, was detailed. It had been shown in continuation of previous work in India (Acton and Knowles, 1915, Caus, Iyengar, and Anderson, 1924) that the effective principle in the immune horse serum was in the pseudoglobulin fraction and that purification and concentration of antivenene was possible by the ammonium sulphate process employed by Maxtrumia *et al* (1930) in fractionation of serum proteins.

Further work has now been done with the object of finding out a suitable technique for concentrating serum for issue on a large scale which would result in the least possible loss of unitage during the process.

The unconcentrated antivenene, as at present issued, is standardized to neutralize not less than 10 milligram of daboia venom and 0.5 milligram of cobra venom per cubic centimetre. The experimental concentration of successive small quantities was carried out on batches of time-expired sera (sera kept at room temperature for over 2 years) and it was found that in all cases the actual daboia titre, which was taken as the index of potency, was much above the declared minimum titre—quantities of the sera varying from 0.6 c.c. to 0.2 c.c. protecting pigeons of standard weight against a dose of 1 milligram daboia venom (10 M L D per pigeon).

A few preliminary trials of concentration were made by the method described by Grasset (1932). According to this method an initial precipitation of euglobulin is carried out by 11.5 per cent saturation with anhydrous sodium sulphate, the

precipitate is separated by filtration and discarded. The filtrate is further saturated with an additional 6.5 per cent anhydrous sodium sulphate when the pseudoglobulin of the serum containing the antivenomous principle separates out leaving the useless albumin in the filtrate. The pseudoglobulin thus collected is left in the refrigerator overnight to allow the greater proportion of the sulphate to crystallize out. The viscid pseudoglobulin separated from the sulphate is then dialysed in cellophane bags to get rid of all traces of the salt. This method, although yielding satisfactory concentration in occasional experiments, failed to give consistent and comparable results in successive batches. Besides that, the loss of unitage during concentration was considerable, being 20 per cent to 60 per cent of the original unitage of unconcentrated sera used in the process.

Further work was then carried out by a longer sodium sulphate process following in its main lines methods of concentration which has been successfully employed for other antitoxic sera.

The procedure adopted was as follows —

- (a) Two volumes of distilled water added to one volume of serum,
- (b) Anhydrous sodium sulphate added in fractions until the specific gravity of the mixture reached 1.175 at 33°C (about 22 per cent sodium sulphate),
- (c) Solution filtered through Whatman filter-paper No. 50, the euglobulin and pseudoglobulin precipitate being retained and the albumin passing through,
- (d) Filter-paper with the precipitate washed in saturated sodium chloride solution to dissolve pseudoglobulin,
- (e) Solution filtered, pseudoglobulin passing through,
- (f) Pseudoglobulin precipitated by the addition of 0.25 per cent acetic acid,
- (g) Precipitate collected on filter-paper and pressed dry,
- (h) Two per cent sodium carbonate mixed with pseudoglobulin and the mixture dialysed in running water until free of sulphate,
- (i) Reaction adjusted to pH 7.4 and 1 per cent sodium chloride and 0.35 per cent trikresol (or other preservative) added,
- (j) Filtration through Seitz filter.

While these are the general lines of the process, minor variations were tried with the following results in successive batches —

BATCH I—tested on 25th January, 1933

Antivenomous serum used was manufactured in 1930

Process Serum diluted with twice its volume of distilled water brought to 33°C, anhydrous sodium sulphate gradually added to bring the solution to sp. gr. 1.175 at 33°C, filtered, precipitate dissolved in saturated salt solution, after standing for one hour at 33°C filtered, to the filtrate 0.25 per cent acetic acid added to precipitate pseudoglobulin, filtered, precipitate pressed to a cheesy mass, 0.3 per cent sodium carbonate added and dialysed in running tap-water. The dialysate although free of sulphate contained jelly-like lumps of pseudoglobulin which did not go into solution even after further

dialysis Its pH was adjusted to 7.2 and 1 per cent sodium chloride added, the lumps were removed by filtration through cotton-wool

Unconcentrated serum

Amount used—500 c.c.

Titre—0.6 c.c. neutralized 1 mg. daboia venom

Total unitage—533

Concentrated serum

Amount recovered—112 c.c. excluding lumps

Titre—0.15 c.c. neutralized 1 mg. daboia venom

Concentration—fourfold

Total unitage—746

Loss of unitage—slightly over 10 per cent

The concentrated serum was treated with 0.35 per cent trikresol passed through Seitz filter and re-tested for potency after storage for 6 days in the refrigerator

Unconcentrated serum

Titre—0.6 c.c. neutralized 1 mg. daboia venom

Titre—1 c.c. neutralized 0.8 mg. cobra venom

(M.L.D. of cobra venom being 0.3 mg.)

Concentrated serum

Titre—0.15 c.c. neutralized 1 mg. daboia venom

Titre—0.25 c.c. neutralized 0.8 mg. cobra venom

Comment

0.3 per cent sodium carbonate which was added to pseudoglobulin precipitate was not sufficient as the latter did not go into complete solution during dialysis

BATCH II—tested on 1st February, 1933

Serum used was manufactured in January 1931

Process Serum diluted with twice its volume of distilled water and brought to 33°C, anhydrous sodium sulphate added to bring the solution to sp. gr. 1.175 at 33°C, filtered, precipitate dissolved in saturated solution of sodium chloride, allowed to stand for 15 minutes at 33°C and filtered, precipitate pressed to a cheesy mass. 3 per cent sodium carbonate added and dialysed in running tap-water for 24 hours. As the dialysate in the bag was turbid it was further dialysed in 1 per cent saline for 48 hours. Dialysate was clear and free of sulphate. To it was added chinosol (1 in 1,000) after the pH had been adjusted to 7.6 and the concentrate passed through a Seitz filter.

Unconcentrated serum

Amount used—200 c.c.

Titre—0.4 c.c. neutralized 1 mg. daboia venom

Total unitage—500

Concentrated serum

Amount recovered—60 c.c.

Titre—0.3 c.c. neutralized 1 mg. of daboia venom

Concentration—1.3 fold

Total unitage—200

Loss of unitage—60 per cent

Comment

(a) 3 per cent sodium carbonate added to the precipitate was sufficient to bring it to solution during dialysis

(b) Dialysis in 1 per cent saline resulted in a loss of potency and a considerable loss in total unitage

BATCH III—tested on 7th February, 1933

Serum used was manufactured in April 1931

Process Serum diluted with twice its volume of distilled water and brought to 33°C, anhydrous sodium sulphate added to bring the solution to sp gr 1175 at 33°C, precipitate dissolved in saturated saline, allowed to stand for 30 minutes at 33°C and filtered, 0.25 per cent acetic acid added to filtrate, re-filtered, precipitate pressed to a cheesy mass and 2 per cent sodium carbonate added, dialysed in running tap-water for 24 hours and in 1 per cent saline for 48 hours. The dialysate was clear. The reaction was adjusted to pH 7.5 and 0.35 per cent trikresol added and concentrate passed through a Sertz filter.

The euglobulin precipitate recovered from the saturated saline was pressed to a cheesy mass and dialysed in 1 per cent saline solution. Its pH was adjusted to 7.0 and 0.35 per cent trikresol added. The concentrate was then passed through a Sertz filter.

<i>Unconcentrated serum</i>	<i>Concentrated serum</i>
Amount used—100 c c	Amount of pseudoglobulin solution recovered—47 c c
	Amount of euglobulin solution recovered 9.5 c c
Titre—0.45 c c neutralized 1 mg daboia venom	Titre of pseudoglobulin fraction—0.4 c c neutralized 1 mg daboia venom
	Titre of euglobulin fraction—0.4 c c did not neutralize 1 mg daboia venom
	Concentration—practically nil
Total unitage—222	Total unitage—117
	Loss of unitage—47 per cent

Comment

Loss of unitage and poor concentration were due to dialysis in 1 per cent salt solution.

BATCH IV—tested on 8th February, 1933

Serum used was same as that employed for Batch III

Process Serum diluted with twice its volume of distilled water and brought to 33°C, anhydrous sodium sulphate added to bring the solution to sp gr 1175 at 33°C, filtered, precipitate dissolved in sodium sulphate solution of sp gr 1175 at 33°C and filtered again, precipitate pressed to a cheesy mass and dialysed in running tap-water for 24 hours and then in 1 per cent salt solution for another 24 hours. The dialysate was free of sulphate. To this 0.35 per cent trikresol was added and filtration through Sertz filter carried out.

<i>Unconcentrated serum</i>	<i>Concentrated globulins</i> (Euglobulin and pseudoglobulin)
Amount used—120 c c	Amount recovered—36 c c after dialysis
Titre—0.45 c c neutralized 1 mg daboia venom	Titre—0.15 c c neutralized 1 mg daboia venom
	Concentration—threefold
Total unitage—266	Total unitage—240
Loss of unitage—less than 10 per cent	

Comment

Dialysis of both fractions together resulted in minimum loss of unitage.

BATCH V—tested on 16th February, 1933

Serum used was manufactured in May 1931

Process 180 c c distilled water added to 120 c c serum, mixture heated to 33°C, anhydrous sodium sulphate gradually added to bring the solution to sp gr 1175 at 33°C, allowed to stand at this temperature for one hour and filtered, precipitate washed in sodium sulphate solution of sp gr 1175 at 32°C and re-filtered, precipitate pressed to a cheesy consistency and dialysed in running tap-water for 24 hours and then in 1 per cent salt solution till free of sulphate. The pH of the dialysate was adjusted to 7.2 and 0.35 per cent trikresol added and filtration through Seitz filter carried out.

Unconcentrated serum

Amount used—120 c c

Titre—Not estimated

Concentrated globulins

Amount recovered—36 c c

Titre—0.15 c c neutralized 1 mg of daboia venom

BATCH VI—tested on 2nd March, 1933

Serum used was that obtained from several laboratory test samples of antivenin manufactured during 1930-1931.

Process Serum diluted with twice its volume of distilled water and brought to 33°C, anhydrous sodium sulphate added to bring the solution to sp gr 1175 at 33°C and allowed to stand at this temperature for one hour and filtered, precipitate washed with sodium sulphate solution of sp gr 1175 and mixture re-filtered, precipitate then washed in saturated salt solution and left for 5 hours at 33°C and filtered, 0.25 per cent acetic acid added to filtrate to precipitate pseudoglobulin, filtered, precipitate pressed to a cheesy mass, 1.5 per cent sodium carbonate added and dialysed in running tap-water for 24 hours, dialysis continued in 1 per cent saline solution for 48 hours till free of sulphate. The pH of the dialysate was adjusted to 7.6 and the concentrate divided into three equal portions. To one lot 0.35 per cent trikresol was added, to another 1 in 1,000 chinisol and to a third lot 1-5,000 merthiolate, and all filtered through Seitz filters.

Unconcentrated serum

Amount used—450 c c

Titre—0.2 c c neutralized 1 mg daboia venom

Concentrated

Amount recovered—140 c c

Titre—0.09 c c of serum treated with trikresol neutralized 1 mg daboia venom. 0.09 c c of serum treated with merthiolate neutralized 1 mg daboia venom. 0.085 c c of serum treated with chinisol neutralized 1 mg daboia venom.

Concentration—just over twofold

Total unitage—2,250

Total unitage—1,555

Loss of unitage—30 per cent

Comment

Loss of potency and unitage were probably due to prolonged dialysis in 1 per cent saline.

BATCH VII

The serum used was the same as in Batch VI.

Process Serum diluted with twice its volume of distilled water and brought to 33°C, anhydrous sodium sulphate gradually added to bring the solution to the

sp gr 1175 at 32°C and filtered, precipitate washed in saturated solution of sodium chloride and allowed to stand for 30 minutes at 33°C and then filtered, 0.25 per cent acetic acid added to filtrate and then re-filtered, precipitate pressed to a cheesy consistency and weighed. This was divided into two equal lots each weighing 29.6 grammes. To one lot 0.3 per cent sodium carbonate was added and to the other 1 per cent sodium carbonate and both these dialysed in running tap-water for 36 hours. The pH of the dialysates was adjusted to 7.4. It was observed that the addition of only 0.3 per cent sodium carbonate to the precipitate before dialysis resulted in a gelatinous fluid of great viscosity which did not improve by the addition of further quantities of sodium carbonate and therefore this portion was discarded. The one to which 1 per cent sodium carbonate was added dialysed better, but small lumps were present in the dialysing bag. To this 1 per cent sodium chloride and 0.35 per cent trikresol were added and the mixture passed through a Seitz filter.

Unconcentrated serum

Amount used—125 c.c.

Titre—0.2 c.c. neutralized 1 mg. daboia venom

Total unitage—625

Concentrated serum

Amount recovered—32 c.c.

Titre—0.07 c.c. neutralized 1 mg. daboia venom

Concentration—about threefold

Total unitage—457

Loss of unitage—27 per cent

Comment

(a) The addition of only 0.3 per cent sodium carbonate to the pseudoglobulin precipitated with acetic acid was insufficient to cause complete solution and a gelatinous fluid with lumps resulted.

(b) The addition of 1 per cent sodium carbonate to the above precipitate before dialysis was also insufficient to prevent the occurrence of gelatinous lumps.

(c) The increased loss of unitage was due to formation of lumps during dialysis which would not go into solution.

BATCH VIII

Serum used was the same as that employed in Batch VI.

Process Serum diluted with twice its volume of distilled water and brought to 33°C, anhydrous sodium sulphate gradually added to bring the sp gr to 1175 at 33°C and filtered, precipitate washed with sodium sulphate solution of sp gr 1175 at 33°C, precipitate dissolved in saturated solution of sodium chloride and allowed to stand at room temperature for two hours and filtered, 0.25 per cent acetic acid added to filtrate and re-filtered, precipitate pressed to a cheesy consistency and 2 per cent sodium carbonate added to it and dialysed in running tap-water, pH of the dialysate adjusted to 7.4, 1 per cent sodium chloride and 0.35 per cent trikresol added and filtration through Seitz filter carried out.

Unconcentrated serum

Amount used—250 c.c.

Titre—0.2 c.c. neutralized 1 mg. daboia venom

Total unitage—1,250

Concentrated serum

Amount recovered—72 c.c.

Titre—0.07 c.c. neutralized 1 mg. daboia venom

Concentration—about threefold

Total unitage—1,023

Loss of unitage—18 per cent

Comment

Two per cent sodium carbonate was just sufficient to prevent the formation of lumps during dialysis

BATCH IX

Sera concentrated by Grasset's method which showed low potency were pooled together and the resulting mixture was diluted with ten times its volume of distilled water. The diluted product was saturated with sodium chloride and left over for a week in the refrigerator. It was then filtered. To the filtrate 0.25 per cent acetic acid was added and the precipitate filtered at room temperature. To the precipitate 3 per cent sodium carbonate was added and dialysed in running tap-water for 36 hours—free of sulphate. The pH of the dialysate was adjusted to 7.4. 1 per cent sodium chloride and 0.35 per cent trikresol added and filtered through a Seitz filter.

Concentrated serum

Amount used—250 c c

Titre—0.4 c c did not neutralize 1 mg daboia venom

(Titre of unconcentrated serum 0.2 c c neutralized 1 mg daboia venom)

Reconcentrated serum

Amount recovered—134 c c

Titre—0.06 c c neutralized 1 mg daboia venom

BATCH X—tested on 26th March, 1933

A further batch was concentrated by methods based on the experience of the previous concentrations allowing a longer period for each stage of precipitation and using the quantities of sodium sulphate and sodium carbonate which had been found to give optimum results.

The serum used was only 3 weeks old

Process Serum diluted with twice its volume of tap-water and brought to 33°C, anhydrous sodium sulphate added to bring the solution to sp gr 1.175 at 32°C and filtered, precipitate washed in saturated solution of sodium chloride and allowed to stand for 2 hours at 33°C, filtered, 0.25 per cent acetic acid added to filtrate and re-filtered through chain cloth, precipitate pressed to a cheesy mass within folds of filter-paper and absorbent twill cloth, two per cent sodium carbonate added to pressed precipitate and dialysed in running tap-water for 18 hours. After adjusting pH to 7.4 and addition of 1 per cent sodium chloride the concentrate was passed through a Seitz filter.

Unconcentrated serum

Amount used—700 c c

Titre—0.5 c c neutralized 1 mg daboia venom

Total unitage—1,400

Concentrated serum

Amount recovered—115 c c

Titre—0.1 c c neutralized 1 mg daboia venom

Concentration—fivefold

Total unitage—1,150

Loss of unitage—18 per cent

Comment

Fivefold concentration was easily obtained from an original serum of comparatively low potency

DISCUSSION

The precise distribution of antivenomous principle in the pseudoglobulin fraction of serum having been established by previous workers it became possible at first to concentrate a few batches by fractionation with ammonium sulphate but in these experiments not more than twofold concentration was obtained. The method was found to have certain drawbacks, e.g., it required a very prolonged dialysis to get rid of all ammonium salts entailing considerable loss of unitage in the final product probably due to bacterial decomposition. In fractionating with sodium sulphate in the present series of experimental batches it was found that the dialysis of the salt in running tap-water was as a rule complete in 12-18 hours and loss of unitage even in small batches was not over 20 per cent. The final product after bacteria-free filtration is much clearer and threefold concentration was easily obtained. With increased experience and the use of larger quantities of serum at a time it is expected that the loss of unitage can be reduced still further and the potency regularly raised to four or fivefold. If these hopes are realized and potency of the concentrate is maintained at least for a year under ordinary conditions of storage the method would be suitable for the manufacture and issue of a potent and purified antivenom on a large scale.

SUMMARY

1 With the sodium sulphate method at least a threefold concentration of antivenomous serum can be obtained.

2 0.25 per cent acetic acid was found to precipitate all the pseudoglobulin from the brine filtrate.

3 Dialysis of the pseudoglobulin precipitate with the addition of 0.3 per cent and 1 per cent sodium carbonate resulted in a lumpy dialysate which did not go into solution by further addition of the alkali. Dialysis of the pseudoglobulin precipitate with the addition of 3 per cent sodium carbonate resulted in a clear liquid dialysate of good appearance.

4 The quantities of the sodium carbonate added to the pseudoglobulin precipitate for dialysis, namely, 0.3, 1, 2 and 3 per cent appeared to have no appreciable effect on the potency of the concentrated serum.

5 Dialysing the pseudoglobulin precipitate in 1 per cent sodium chloride solution resulted in a product of low potency with considerable loss in unitage.

6 The addition of 0.35 per cent trikresol, 1 in 1,000 chinolol or 1 in 5,000 merthiolate had no adverse influence on the antivenomous potency of the concentrated serum, when tested one week after their addition.

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AN IMPROVED MICRO-METHOD OF ESTIMATING IODINE

BY

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VARIOUS micro-methods of estimating iodine have been devised, but none of them take sufficiently into consideration the various extraneous factors which interfere with the estimation. The methods in use by different workers are either titrimetric or colorimetric. In the titrimetric methods one of the two courses is followed (a) the iodide is oxidized to iodate either by chlorine (Fellenberg, 1924), bromine (Kendall, 1920, Kelly and Husband, 1924, Leitch and Henderson, 1926, Hercus, Aitken, Thomson and Cox, 1931, Taylor, 1931), or by alkaline permanganate (Gerard and Raunnet, 1932, Grutzner, 1914), iodine-free potassium iodide in acid medium being subsequently added and the liberated iodine titrated with thiosulphate using starch as indicator, or (b) the iodine is liberated from the iodide by means of nitrosyl-sulphuric acid, absorbed in carbon disulphide and titrated in acid-free medium with thiosulphate (Hercus and Roberts, 1925).

The colorimetric methods depend on liberating the iodine from the iodide with nitrosyl-sulphuric acid (Fellenberg, 1924, Newcomb and Sankaran, 1930, McClendon, 1924) or nitrite sulphuric acid reagent (Hercus and Roberts, 1927) and comparing the colour given by iodine in carbon disulphide, chloroform or carbon tetrachloride, with standards, either by naked-eye approximation or by a micro-colorimeter. The partition coefficient of iodine between carbon disulphide and water is 620, between chloroform and water, 130, and between carbon tetrachloride and water, 87. Hence, carbon disulphide was used as a solvent for the iodine.

In all these methods the loss of iodine due to incineration, in the process of converting the organically combined iodine into the alkali iodide, is considerable, so also is the risk of direct or indirect contamination of the material under analysis by iodine. It was therefore thought desirable to examine the accuracy of these methods and, if possible, to devise one in

which the margin of experimental error was reduced to a lower limit To this end the following experiments were made —

Experiments : GROUP I

Fellenberg's method, as adopted by Newcomb and Sankaran (1930), was used in an attempt to determine the conditions for the optimum yield of iodine in human urine (U_1) stocked in bottles with potassium carbonate

(a) *To ascertain the optimum time of evaporation, the time of incineration and fusion being kept as 30 minutes*

The following observations were made, the samples being incinerated at below red-heat temperature, approximately 300°C --

Observation number	Volume of urine taken in c c	Time of evaporation in hours	Iodine recovered in γ
1	50	1	20
2	50	2	20
3	50	3	20
4	50	1	20
5	50	2	20
6	50	2	20
7	50	3	20
8	50	3	20
9	50	1	20

These observations show that there is no change in the recovery of iodine with the time of evaporation The optimum time of evaporation is therefore one hour

(b) To ascertain the optimum time of incineration and fusion, the time of evaporation being kept at one hour

The following observations were made —

Observation number	Volume of urine taken in c c	Time of incineration and fusion in minutes	Fused mass powdered or not	Iodine recovered in γ
10	50	5	Not powdered	10
11	50	10	„	10
12	50	10	Powdered	15
13	50	15	„	20
14	50	20	„	25
15	50	25	Not powdered	20
16	50	30	Powdered	20
17	50	30	„	20
18	50	60	„	15
19	50	20	„	25
20	50	120	„	15
21	50	25	„	25

These observations show that the optimum time of fusion varies from 20 to 25 minutes. If the fused mass is not powdered thoroughly at the stage of incineration there is a fall in the recovery of iodine. The optimum time of fusion is that at which all the organic matter is disorganized at about 300°C, viz., 20 to 25 minutes.

The following is the detail of conditions found to be optimum for the recovery of iodine from samples of urine, when Fellenberg's method is used —

Evaporate over a Fisher burner a mixture of 50 c c of urine, 2 g of K_2CO_3 and 2 c c of 10 per cent potash with constant stirring in a nickel dish, the evaporation to be completed in one hour. Slowly ignite and incinerate at below red-heat for about 20 minutes. Meanwhile thoroughly powder the mass with a glass-rod. By this time all the aromatic smell will have disappeared.

After cooling add about 5 c c of water, evaporate to a pasty condition and again cool. Add 2 c c of 80 per cent alcohol, mix well with a glass-rod and transfer the alcoholic solution of potassium iodide to a small nickel dish. If the paste tends to run over with the alcohol, extract several times with 0.5 c c of absolute alcohol till the pasty mass is restored to its proper consistency. Repeat the extraction with 80 per cent and absolute alcohol till the volume of the extract is about 5 c c. Add to this extract one drop of ten per cent potash and evaporate to dryness over a water-bath. The residue is then whitish. Gently heat over a free flame till the

residue begins to fuse. The residue then appears slightly charred. Cool and extract with 0.2 c.c. of 80 per cent and absolute alcohol till the volume of the extract in a platinum dish is about 1 c.c. Add one drop of 10 per cent potash and evaporate to dryness over the water-bath. Wave the dish over a naked flame till the solid residue just begins to melt. Then cool and add three drops of distilled water, mix with a small glass-rod and transfer the solution to a clean and dry shaking-out tube. Wash the platinum dish three or four times, using two drops of distilled water for each washing, and add the washings to the shaking-out tube. The volume of the wash in the tube will then be about 0.4 c.c. To this colourless solution add one drop of decinormal sodium arsenite solution and a few drops (usually two) of 5 N sulphuric acid till acid, allow to stand for five minutes in order that the reduction of iodate into iodide may be completed. Then add one drop of carbon disulphide and one drop of nitrosyl-sulphuric acid, shake well and centrifugalize. If the colour is very intense add some more carbon disulphide, shake and centrifugalize. Then compare the colour with that of standards.

Experiments : GROUP II

The following control analyses were made by the above method in order to determine the extent of the loss of iodine consequent on incineration, and to compare any such loss with that occurring in the methods devised by other workers —

(a) Using 50 c.c. of stock urine (U_2) with or without the addition of iodine in the form of potassium iodide the following results were obtained —

Observation number	Volume of urine taken in c.c.	Iodine added in γ	Iodine recovered in γ	Mean recovery of iodine in γ	Percentage of loss
22	50	0	2.0	2.0	"
23	50	0	2.0		
24	50	0	2.0		
25	50	1.0	2.6	2.6	40.0
26	50	1.0	2.6		
27	50	1.0	2.6		
28	50	2.0	3.5	3.5	25.0
29	50	2.0	3.5		
30	50	2.0	3.5		

These observations show that the loss of iodine by this method varies from 25 to 40 per cent. The amount of iodine recovered is not an absolute measure of the amount of iodine present in the urine. It indicates only the amount that can be estimated by this method.

(b) Known amounts of iodine were added to the residue after incineration, and the recovery of iodine noted, to see if the loss of iodine observed in the previous experiment was due to liberation of smoke or to imperfect extraction. With a sample of urine (U_3) the following observations were made —

Obser vation number	Volume of urine taken in c c	Iodine added in γ	Iodine recovered in γ	Mean recovery of iodine in γ	Percentage of loss
31	50	0.0	2.2	2.33	80
32	50	0.0	2.4		
33	50	0.0	2.4		
34	50	2.0	4.0	4.17	
35	50	2.0	4.0		
36	50	2.0	4.5		

These observations show that the loss consequent on imperfect extraction is 8 per cent, that due to the incineration in II (a) seems to vary from 17 to 32 per cent.

(c) Iodine was added to the incinerated samples of urine (U_3), filtered through a Whatman filter, No. 44, using 95 per cent alcohol, and the recovery noted. The object of this experiment was to see if there is any loss during filtration in the process adopted by Leitch and Henderson (1926). The following observations were made —

Observation number	Volume of U_3 taken in c c	Iodine added in γ	Iodine recovered in γ	Mean recovery of iodine in γ	Percentage of loss
37	50	1.0	3.2	3.27	6.6
38	50	1.0	3.4		
39	50	1.0	3.2		

These observations show that there is very little loss during filtration with alcohol.

(d) Iodine was added to the incinerated samples of urine (U_3) as in experiment II (c) but in the filtration process hot water was used instead of 95 per cent alcohol. The object of this experiment was to see if there was any loss during

filtration in the process adopted by Heicus and Roberts (1927) The following observations were made —

Observation number	Volume of U_2 taken in c c	Iodine added in γ	Iodine recovered in γ	Mean recovery of iodine in γ	Percentage of loss
40	50	20	38	39	21.5
41	50	20	38		
42	50	20	40		
43	50	10	30	31	23.0
44	50	10	32		

These observations show that there is an appreciable loss in the recovery of iodine amounting to about 22 per cent on an average

(e) Four Whatman No 1 filter-papers were soaked with known quantities of iodine, boiled with water and filtered, the filtrate was evaporated to dryness, extracted with alcohol, and the iodine estimated as usual. The object of this experiment was to see if there is any loss in the process adopted by Baumann and Nannette (1932) The following observations were made —

Observation number	Number of filter papers taken	Iodine added in γ	Iodine recovered in γ	Mean recovery of iodine in γ	Percentage of loss
45	4	25	20	21.3	14.8
46	4	25	22		
47	4	25	22		

These observations show that there was a fall in the recovery of iodine of approximately 15 per cent

(f) Known quantities of iodine were added to varying amounts of unwashed rice-powder and the recovery of iodine noted. The object of this experiment was to see if the loss of iodine is dependent on the amount of smoke evolved during incineration. The following observations were made —

Observation number	Amount of rice powder taken in grammes	Iodine added in γ	Iodine recovered in γ	Percentage of loss
48	10	25	0.8	66
49	25	25	0.6	76
50	50	25	0.5	80
51	50	0.0	0.0	

These observations show that the greater the evolution of smoke the greater is the loss of iodine during incineration

Experiments . GROUP III

The following results indicate the importance of pH at the critical sensitiveness of starch-iodide colour and the extent to which the accuracy of titrimetric methods depends on the inter-relationship between pH and the limitations of starch-iodide colour —

Observation number	Free I_2 in 10 c c	Amount of acid in 10 c c	Strength of colour	pH	Recovery of iodine in γ
52	30	<i>Nil</i>	Very faint blue	2.7	28
53	30	1 c c N/100 H_2SO_4	Faint blue	2.2	32
54	30	1 c c N/10 H_2SO_4	Faint blue	2.0	30
55	30	1 c c N H_2SO_4	Blue	1.2	38
56	30	1 c c 10 N H_2SO_4	Blue	<1.2	44
57	20	<i>Nil</i>	<i>Nil</i>	4.7	
58	20	1 c c N/100 H_2SO_4	<i>Nil</i>	4.1	
59	20	1 c c N/10 H_2SO_4	Faint blue	2.2	18
60	20	1 c c N H_2SO_4	Faint blue	2.0	24
61	20	1 c c 10 N H_2SO_4	Blue	<1.2	36
62	30	0.1 c c 20 per cent H_3PO_4	Blue	2.2	31
63	30	0.5 c c 20 „ H_3PO_4	Deep blue	<1.2	34
64	30	1.0 c c 20 „ H_3PO_4	Deep blue	<1.2	36
65	30	2.0 c c 20 „ H_3PO_4	Deep blue	<1.2	35
66	30	1.0 c c 80 „ H_3PO_4	Deep blue	<1.2	40
67	20	0.1 c c 20 „ H_3PO_4	Very faint blue	3.2	18
68	20	0.5 c c 20 „ H_3PO_4	Faint blue	2.2	22
69	20	1.0 c c 20 „ H_3PO_4	Blue	<1.2	24
70	20	2.0 c c 20 „ H_3PO_4	Blue	<1.2	24
71	20	1.0 c c 80 „ H_3PO_4	Blue	<1.2	30

Notes — (1) By 'Free I_2 ' is meant a solution of iodine in potassium iodide

(2) Kahlbaum H_2SO_4 and H_3PO_4 were used.

(3) The pH determinations were made by the glass electrode (Sankaran, 1933) after the addition of two drops of one per cent solution of soluble starch

(4) The recovery of iodine was determined by titration from the duplicate solutions

These results show that the colour of starch-iodide remains steady near the end-point with a very faint blue tinge even after the addition of thiosulphate equivalent to 2γ of iodine. There is therefore an inevitable over-titration. The starch-iodide colour increases with the concentration of hydrogen ions.

Discussion of results of the experiments.

It was found from experiments I (a) and I (b) that as much as 40 per cent of the iodine could be lost during a prolonged incineration of two hours even though the temperature at which the incineration was carried out was as low as 300°C. Further, if the sample was not thoroughly incinerated then the recovery was as low as 60 per cent or less. The best results were obtained when the sample was incinerated free from organic matter at low heat.

The results of experiment II (a) show that as much as 0.4γ may be lost from 1.0γ of added iodine, and as much as 0.5γ from 2.0γ of added iodine during incineration. As 0.4γ and 0.5γ can be taken as nearly equal, the loss is not a percentage one, but an absolute one depending on the amount of organic matter present. From experiments II (b) and II (c) it is seen that the error during the process of extraction of the potassium iodide from the incinerated sample is nearly the same in the method of Fellenberg as in that of Leitch and Henderson wherein the potassium iodide is extracted with 95 per cent alcohol and filtered through a Whatman No. 44. Experiments II (d) and II (e) indicate that there is an appreciable retention of iodide by the filter-papers used in the methods of Hercus and Roberts (1927) and of Baumann and Nannette (1932). The results of experiment II (f) show that the loss of iodine mainly depends on the amount of smoke evolved—the greater the amount of smoke the greater is the loss. In experiment II (f) as much as 5 c.c. of 10 per cent potash was used for every gramme of the rice-powder. The loss of iodine is not therefore due to the want of alkali but is due to the liberation of smoke in which activated carbon in the form of a stable aerosol carries off some KI.

From experiment III it is seen that the starch-iodide colour increases with the concentration of hydrogen ions resulting in a higher yield of iodine. The pH should be at 2.2 in order that a satisfactory recovery of iodine may be made when the iodine content in the phosphoric acid medium for titration is not less than 2.0γ per c.c. The end-point of starch-iodide being unsatisfactory, over-titration is found to be inevitable in most cases.

In all the micro-titrimetric methods, except that of Hercus and Roberts (1925), a great amount of KI is added in order to liberate the iodine in acid medium according to the reaction— $5\text{HI} + \text{HIO}_3 = 3\text{H}_2\text{O} + 3\text{I}_2$. This method evidently increases the iodine content of a sample by six times but the sensitiveness of the method is masked by the contamination of iodine from the KI and the decomposition of thiosulphate in acid medium. The Kahlbaum sample of KI which had been heated in an air oven for seven hours at 110°C and then desiccated overnight was found to contain as much as 4γ per c.c. of a freshly prepared 10 per cent solution of KI. The amount of free iodine then increases on standing, turning the solution yellow. This is due to the fact that the solution of KI oxidizes in the air with the formation of hypoiodite which in the presence of water liberates iodine according to the reaction— $2\text{KIO} + \text{H}_2\text{O} = 2\text{KOH} + \text{I}_2$. Even solid KI has a

tendency to form KIO in the presence of sunlight, or of traces of occluded oxidizing agents such as Cl, Br or atmospheric oxygen

Secondly, the titration of iodine by means of thiosulphate is made only in acid medium in which part of the thio-sulphate is decomposed into sulphurdioxide, sulphur and polythionates. The colloidal sulphur formed in the medium from the dilute thiosulphate coagulates slowly into a turbid state, though at the same time the decomposition of the thio-salt is in no way less. The solution remains clear for a longer time in acid medium if alcohol is added because the sulphur solution is more stable in alcoholic than in aqueous solution. So the addition of amyl alcohol to sodium thiosulphate solution by Heicus *et al* (1931) retards the flocculation of sulphur from the decomposed thio salt in acid solution rather than stabilizes it. In all the titrimetric methods of micro estimations of iodine factors are involved which are not always capable of adequate control. Amongst them are (1) the partial decomposition of sodium thiosulphate in acid medium, (2) the development of starch-iodide colour resulting in a higher recovery of iodine at pH less than 2.2, and the limitations of starch iodide colour at pH greater than 2.2. These factors tend to increase the margin of experimental error. While determining 1% to 4% of I_2 Baumann and Nannette add as much as 60.0 mg of KI, and for estimating 5% to 30% they use up to 150 mg of KI to get 93 to 100 per cent recovery. Allott, Dauphinee and Hurtle (1932) in the estimation of 2% of I_2 use 1,000 to 2,000% of KI to get 99 to 101 per cent recovery. It has also been determined by the latter workers that the addition of lower amounts of KI gave lower yields and that higher amounts gave higher values. It is therefore clear that in all the micro-titrimetric control experiments the addition of KI and the acid concentration are so manipulated as to liberate as much of iodine from the KI as roughly to neutralize the errors.

It was therefore found necessary to improvise a colorimetric method in which the errors due to the liberation of smoke and the presence of reducing agents are eliminated or reduced to the minimum. The method is as follows —

An improved method of iodine estimation

The sample for analysis is rendered alkaline. Fats and oils should first be saponified with 10 per cent alcoholic potash, then evaporated to dryness in a porcelain basin and dissolved in hot water. Cereals and vegetables should be heated with alkali till a complete paste is obtained. Whatever the sample may be it should be converted into an easily workable form. To such an alkaline solution 7 per cent solution of $KMnO_4$ is gradually added, and while stirring with a glass-rod the porcelain basin is heated over an asbestos protected flame. When the oxidation is complete and there is a slight excess of $KMnO_4$, absolute alcohol is added slowly till the excess of $KMnO_4$ is reduced. It is then cooled and filtered quantitatively and the hydrated manganese dioxide removed. The filtrate is then evaporated to dryness with a small amount of K_2CO_3 . The residue which always contains some non-oxidizable disintegration product, besides potassium iodate, is heated over a protected flame till charring takes place without the evolution of smoke*. It is then heated slowly over a mild flame till the organic

* Some amount of smoke is evolved from fats and oils at this stage—the amount of smoke being very much less than that in the usual incineration method

matter is disorganized. During this process of heating all the iodate coming in contact with the finely divided carbon is reduced to iodide. It is then made into a paste and extracted and estimated as usual.

If during incineration the charred mass remains in a molten state due to an excess of potash, a requisite amount of K_2CO_3 should be added to render the final product suitable for alcoholic extraction.

For the determination of iodine from pure $KMnO_4$, 50 g of $KMnO_4$ is taken, and after reduction with alcohol it is filtered quantitatively. The filtrate is evaporated in five nickel dishes after addition of 3 g of K_2CO_3 to each dish. Then before the evaporation is complete one gramme of sugar (there being no iodine in sugar) is distributed amongst the five dishes. The rest of the process remains the same. In every determination the amount of iodine present in the $KMnO_4$ used up is subtracted in order to get the actual value.

A few control analysis of urines have been made by this method and the results are given below —

Obser- vation number	Volume of urine taken in c c (rat's urine)	Iodine added in γ	Iodine recovered in γ
72	25	0 0	0 8
73	25	1 0	1 6
74	25	2 0	2 5
75	25	1 0	1 5
76	25	1 5	2 0
77	25	1 0	1 6
78	25	1 5	2 0
79	25	3 0	3 8
80	25	1 0	1 5

These observations show that the average recovery of iodine was 88.7 per cent. The recovery was only 75 per cent when 1.0 γ of iodine was added, but it increased with increasing amount of iodine added, so much so that when 3.0 γ was added the recovery was quantitative. It therefore appears that if samples were so chosen as to contain between 1.0 γ and 3.0 γ of iodine the estimated values would represent 88.7 per cent of what would be actually contained in the sample. Attempts were made, as far as possible, to so choose samples as to contain 1.0 γ to 3.0 γ of iodine, and in such estimations the observed value was multiplied by $\frac{100}{88.7}$. But with certain food-stuffs containing very little iodine, a greater bulk had to be taken. To avoid the tediousness of handling too large a bulk of the material, estimations were made on convenient samples and the results observed reported. It should be mentioned that such values are bound to be too low, especially as the error is considerable in estimations below 1.0 γ of iodine.

The following experiments were then carried out in order to compare the recovery of iodine by both the old and the improved methods. The samples

yielding less than 10% of iodine from convenient samples have been marked in asterisks(*) —

Observation number	Sample	Recovery by the improved method (observed values)	Recovery by the old method (observed values)
S1	Urine U ₁	48 γ per litre	32 γ per litre
S2	" U ₂	64 γ "	48 γ "
S3	" U ₃	60 γ "	48 γ "
S4	" U ₄	60 γ "	40 γ "
S5	" U ₅	76 γ "	64 γ "
S6	Thyroid solution (mean of 4)	185 γ "	175 γ "
S7	Milk (mean of 3)	353 γ "	162 γ "
S8	Superior diet (Coonoor stock rats)	34 γ per kg	16 γ per kg
S9	White flour	58 γ "	60 γ "
90	Unwashed rice	28 γ "	120 γ "
91	Ragi	90 γ "	160 γ "
92	Washed rice	18 γ "	80 γ "
93	Wheat flour	24 γ "	40 γ "
94	Cod liver oil	4,920 γ per litre	4,400 γ per litre
*95	Cotton seed oil	75 γ "	0 γ "
96	Grass (green)	1706 γ per kg	100 γ per kg
97	Shorgum	260 γ "	16 γ "
*98	Yeast	80 γ	4 γ "
99	Orange juice	53 γ per litre	4 γ per litre
*100	Onion	60 γ per kg	30 γ per kg
*101	Carrot	90 γ "	60 γ "
102	Butter	276 γ "	100 γ "
*103	Cabbage, innermost leaves (Mean of 5)	77 γ 84 γ	60 γ "
104	Cabbage, outermost leaves (Mean of 6)	547 γ 689 γ	400 γ "
*105	Ground nut	54 γ "	Trace (2 γ per kg)

Comparing the results yielded by the two methods it is apparent that the estimated amounts by the improved method are very much higher than those by the old method

Summary.

(1) Fellenberg's method, as adopted by Newcomb and Sankaran has been standardized to give an optimum yield of iodine

(2) It has been shown that the loss in the colorimetric methods of estimating iodine is mainly due to the evolution of smoke during the process of incineration

(3) It has been shown also that various extraneous factors, incapable of adequate control, tend to introduce a high degree of error into the titrimetric methods

(4) An improvised method, which to a great extent eliminates the errors due to the evolution of smoke and reducing matter, has been described, the yield of iodine by this method amounts to 88.7 per cent of iodine content of the material analysed, and comparative data for the recovery of iodine both by the old and by the new method have been given

In conclusion I desire to express my indebtedness to Colonel Sir Robert McCarrison, Dr G Sankaran and Mr S Ranganathan for their kind help and guidance during the course of this work

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PHARMACOLOGICAL ACTION OF CERTAIN DERIVATIVES OF COTARNINE

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CONSIDERABLE quantities of narcotine are obtained as a by-product in the manufacture of morphine. At the request of the Central Board of Revenue, Government of India, attempts were made to make use of this alkaloid by converting it into such compounds as may have anti-malarial and anti-pyretic properties. The details of the preparation of some of these derivatives have been already published by Ahluwalia, Kochhar and Ray (1932). In this paper it is proposed to give a brief account of their pharmacological action.

Action on protozoa — The effects produced by the hydrochlorides of the various condensation products of cotarnine were studied and compared with that of quinine hydrochloride. The following tables give briefly the results obtained —

TABLE I

Showing the comparative effects of different dilutions of 2' 4' dihydroxy-phenyl-hydrocotarnine and quinine hydrochloride on Paramœcium in 24 hours

Dilution	2' 4' dihydroxy phenyl hydro cotarnine hydro chloride	Quinine hydro chloride
1 40,000	All dead	All dead
1 50 000	'	"
1 75,000	'	"
1 100 000	'	"
1 150,000	20 per cent dead	All " alive
1 200 000	All alive	'

TABLE II

Showing the comparative effects of 2' 4' dihydroxy-phenyl-cotarnine-hydrochloride and quinine hydrochloride in higher concentration on Paramœcium

Concentration of the drug used	MOVEMENT BECOMES SLUGGISH		DEATH	
	2' 4' dihydroxy phenyl cotarnine hydrochloride	Quinine hydro chloride	2' 4' dihydroxy phenyl cotarnine hydrochloride	Quinine hydro chloride
1 500	At once	At once	At once	At once
1 1,000		Less than a minute	1 3 minutes	0 6 minute
1 2,000	Less than a minute	1 0 minute	2-2 5 „	1 3 minutes
1 3,000	1 0 minute	1 5 minutes	4 0 „	2 0 „
1 4,000	2 0 minutes	3 0 „	5-6 „	3 5 „
1 8,000	5 0 „	6 0 „	22 0 „	10 0 „
1 16,000	6 7 „	15 0 „	50 0 „	28 33 „

Action on plain muscle—Intestine The method of Magnus was employed, strips of isolated pieces of intestine of the rabbit being used. In strengths varying from 1 100,000 to 1 200,000, 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride lowers the tone of the longitudinal muscle fibres and decreases the amplitude of contractions whereas p-ethoxy-amino-phenyl-hydrocotarnine-hydrochloride slightly augments both.

Uterus—Rabbit's uterus was used in these experiments, contraction produced by adrenaline being used for purposes of comparison. The observations of Kehrer that cotarnine-hydrochloride increases the tone and contractions of isolated strips of both pregnant and non-pregnant uterus was confirmed. It was found that 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride produced immediate contraction resembling adrenaline while cotarnine-hydrochloride is slow in action and is less powerful. The uterus does not relax immediately as it does after adrenaline. P-ethoxy-amino-hydrocotarnine-hydrochloride in equal concentrations shows results similar to cotarnine-hydrochloride.

Circulation and respiration—The effect on the cardiac muscle was studied on intact and isolated frog's heart. Cotarnine-hydrochloride in concentrations of 1 10,000 to 1 50,000 produced gradual increase in the amplitude of the heart-beats without affecting its frequency. 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride in similar dilutions produced diastolic dilatation with decrease of amplitude of the beats. The heart is slowed at first followed later by impairment of conduction of impulses resulting in partial or complete heart block. P-ethoxy-amino-phenyl-cotarnine-hydrochloride resembles cotarnine-hydrochloride in its

action on the cardiac muscle, but its action is more marked, both systole and diastole being increased

The comparative action of these drugs and cotarnine-hydrochloride on the circulation and respiration was studied in cats and dogs. It was found that 4 mg to 8 mg of 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride injected intravenously produced a slight fall in blood-pressure and increased respiratory movements. Both the respiration and blood-pressure, however, quickly return to normal. P-ethoxy-amino-phenyl-hydrocotarnine-hydrochloride and cotarnine-hydrochloride resemble each other in their action on the circulation. They both produce variable changes in blood-pressure sometimes a slight momentary fall and at other times a slight momentary rise.

Hæmolysis—The hæmolytic actions of cotarnine-hydrochloride, 2' 4' dihydroxy-cotarnine-hydrochloride and p-ethoxy-amino-phenyl-hydrocotarnine-hydrochloride were studied on washed red-blood corpuscles of rabbits suspended in a buffered Ringer solution with pH of 7.2. Quinine hydrochloride and saponin were used as control. It was found that cotarnine-hydrochloride had no hæmolytic action in one hour in concentration of 1 : 250, whereas 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride produced complete hæmolysis of red-blood corpuscles in five minutes in a concentration of 1 : 250 and partial hæmolysis in five minutes in a concentration of 1 : 500. P-ethoxy-amino-phenyl-hydrocotarnine-hydrochloride produced in a concentration of 1 : 250 an incomplete hæmolysis in five minutes and complete hæmolysis in ten minutes. In 1 : 500 it produced only incomplete hæmolysis in ten minutes.

Anti-pyretic action—The anti-pyretic effects of cotarnine-hydrochloride, 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride and p-ethoxy-amino-phenyl-hydrocotarnine-hydrochloride were studied by producing experimental pyrexia in rabbits by Kilone's method. This consists briefly in injecting hypodermically 4.5 c.c. of a broth culture of *Bacillus coli communis* which has been grown for four days, and then killed by heating to 65°C for one hour. Five rabbits marked A, B, C, D and E were placed in cages and fed on a diet of green grass and grains, and were given plenty of water to drink. Daily records of the rectal temperature were taken during the day at three-hourly intervals for four days before producing pyrexia by injecting *B. coli* vaccine. The routine of the experiment was as follows. The normal temperature of the rabbits was taken at 9-30 a.m. and after 4.5 c.c. of vaccine was injected subcutaneously the temperature was taken every half hour. The drug to be tested and the control quinine hydrochloride were injected after the temperature had remained constant at least for one hour.

The subsequent set of observations were made at a weekly interval. Drugs were given in rotation to eliminate the source of error due to individual variation in the animal used. It was found that both 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride and p-ethoxy-amino-phenyl-hydrocotarnine-hydrochloride are better anti-pyretics than cotarnine hydrochloride. 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride is slightly better anti-pyretic than p-ethoxy-amino-phenyl-hydrocotarnine-hydrochloride, but neither of the drugs are as potent as quinine hydrochloride.

Toxicity—The minimum lethal dose of 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride and p-ethoxy-amino-phenyl-hydrocotarnine-hydrochloride were

determined on frogs and compared with that of cotarnine-hydrochloride. The drugs were injected in the ventral lymph sac of the frogs. Care was taken to prepare a fresh solution every time. Lethal doses for frog were as follows: Cotarnine-hydrochloride 150 mg per kilo body-weight, 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride 250 mg per kilo body-weight, p-ethoxy-amino-phenyl-cotarnine-hydrochloride 200 mg per kilo body-weight.

The symptoms produced by injecting 2' 4' dihydroxy-phenyl-cotarnine-hydrochloride and p-ethoxy-amino-phenyl-cotarnine-hydrochloride in the lymph sac of frogs are inco-ordination of movement, followed by paralysis and cessation of movements. The frogs lay on their abdomen with limbs fully relaxed. The respiration failed next, but the heart continued beating for a long time. Firm pressure on the toe produced a generalized response in the form of twitching of the lower extremities instead of the usual local response. Direct electrical stimulation of the muscles caused their contraction even after the circulation had ceased.

These symptoms are quite comparable with those of cotarnine-hydrochloride. From an analysis of the symptoms described above it can safely be assumed that these drugs produced progressive depression of the central nervous system from above downwards. The exaggerated reflex, as shown by twitchings of the legs and the body muscles by applying firm pressure on the toe, may either be due to slight stimulation of the spinal cord or due to release action of the cerebrum. We are inclined to believe that this is due to its stimulation of the spinal cord. It has not been ascertained whether the effect is on the sensory or on the motor side of the cord.

SUMMARY

The pharmacological actions of certain condensation products of narcotin with various phenols have been studied.

These compounds are toxic to the *Paramæcium*. 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride arrests the movements of *Paramæcium caudatum* sooner than quinine hydrochloride does. The killing time, however, is longer than quinine.

The plain muscle of the intestines is definitely depressed by these compounds in concentration varying from 1 in 100,000 to 1 in 200,000. One member of the series, p-ethoxy-amino-phenyl-hydrocotarnine-hydrochloride, slightly augments the tone. The contraction and tone of the musculature of both the pregnant and non-pregnant uteri are increased slightly with almost all the members of the series.

The blood-pressure falls as a result of intravenous injection of these drugs. This has been shown to be due mainly to the depressant action on the cardiac musculature. The respiratory movements are slightly increased.

The hæmolytic action has also been studied on rabbits' blood corpuscles. Some cotarnine derivatives like 2' 4' dihydroxy-phenyl-cotarnine-hydrochloride produce complete hæmolysis in 5 minutes in a concentration of 1 in 250. The other compounds of the series produce only an incomplete hæmolysis.

The anti-pyretic effects also vary with the different compounds 2' 4' dihydroxy-phenyl-hydrocotarnine-hydrochloride has been shown to be a slightly better anti-pyretic than p-ethoxy-amino-phenyl-hydrocotarnine-hydrochloride. The drugs of this series, however, are much weaker than quinine. The minimum lethal dose in frogs is from 200 mg to 250 mg per kilo body-weight so that these compounds are not very toxic.

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THE PHARMACOLOGICAL ACTION AND ANTI-MALARIAL
PROPERTIES OF ANHYDROCOTARNINE-
RESORCINOL-HYDROCHLORIDE (A
DERIVATIVE OF NARCOTINE)

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IN the course of evidence before the Opium Commission (1895) it was stated that opium had marked anti-malarial properties. Sir William Robert, the medical member of the Commission, suggested that of the two abundant alkaloids of opium, morphia represented the anodyne and hypnotic properties of the drug, and narcotine was the bitter crystalline alkaloid resembling quinine and like that substance possessed tonic and anti-periodic properties. Previously Palmer (1857-1859) had treated a large number of cases of malaria with narcotine and reported successful results. Later Gordon confirmed the results obtained by Palmer and asserted that narcotine was a good anti-periodic and cured cases of malaria in which quinine had failed. After this work narcotine, being cheap and easily available as a by-product in the manufacture of morphine, was in great demand and was regularly supplied from the Government factories. With the increased production of cinchona alkaloids from the Government plantations and factories, however, the demand for narcotine gradually decreased, although its reputation as an anti-periodic persisted.

Chopra and Knowles (1930) carefully studied the action of narcotine on a series of patients suffering from different types of malaria and came to the conclusion that the drug had neither prophylactic nor curative effect in this disease. It

has, however, been maintained in some quarters that though narcotine itself may not have anti-malarial action, some of its derivatives may develop it. If this turned out to be true, it would be possible to use large quantities of this alkaloid obtained during the manufacture of morphine and codeine, which are at present being wasted. Accordingly a series of compounds were prepared by Ahluwalia, Kochhar and Ray (1932) at the University Chemical Laboratories, Lahore, by oxidizing narcotine to cotarnine and later condensing cotarnine with phenols like phloroglucinol, pyrogallol and resorcinol. The pharmacological action and anti-malarial properties of one of these compounds 2,4-dihydroxy-phenyl-hydriocotarnine, also known as anhydriocotarnine-resorcinol compound, were investigated by the present authors.

This compound is a dull-white crystalline powder, sparingly soluble in water. Solutions in water are slightly acid having a pH ranging between 7.4 and 7.6, they are quite stable and do not change by keeping for two or three days or longer.

Pharmacological action —The pharmacological action of this compound closely resembles narcotine as worked out by Chopra, Mukherjee and Dikshit (1930). It is not necessary, therefore, to describe it in detail.

Action on Paramœcium caudatum —Anhydriocotarnine-resorcinol-hydrochloride is fairly toxic to lower forms of life. In concentrations ranging from 1 in 6,000 to 1 in 10,000 the movements of *Paramœcium* are considerably slowed within 5–7 minutes and death occurs within half an hour. Weaker dilutions do not produce any appreciable effects. Quinine hydrochloride in similar concentrations is slightly more toxic.

Local action —Solutions of the compound have no irritant effect on the skin. A 1 to 2 per cent solution instilled into the eye of a rabbit does not produce any marked local irritation of the conjunctiva. The drug is fairly readily absorbed and injection of a 2 per cent solution into the thigh muscles of a cat does not set up local reaction in form of congestion, œdema or necrosis of the tissues.

Alimentary tract —The peristaltic movements of the small intestine recorded with Jackson's enterograph show a definite and well-marked inhibition. This, however, is a temporary effect and the movements become active again. Isolated pieces of the ileum perfused in a uterine bath show a definite inhibition in concentrations ranging from 1 in 100,000 to 1 in 200,000.

Respiratory system —The respiratory movements in the cat under urethane anaesthesia are definitely stimulated, both the amplitude and frequency being increased after intravenous injections of 10 mg to 15 mg. This effect is probably due to stimulation of the respiratory centre similar to that produced by narcotine.

Circulatory system —Intravenous injections of 5 mg to 15 mg of the drug, in cats under chloralose anaesthesia, show a fall of systemic blood-pressure varying between 15 mm to 20 mm of mercury. This fall is usually of a short duration and is followed by a rise of the pressure slightly above normal level. Smaller doses may produce little or no fall in blood-pressure, but with 20 mg or more irregularities in the blood-pressure curve are frequently observed. This is probably due to spasmodic contractions of the diaphragm produced by the action of the drug on the respiratory centre. The fall in systemic blood-pressure appears to be mainly due to dilatation of the vessels of the splanchnic area. This is

shown by the fact that there is a considerable increase in the volume of such organs as the intestines, spleen, kidneys, etc

The heart—0.5 mg to 1 mg of the drug given intrahepatically or by injections into the lymph sacs of pithed frogs produce a definite slowing of the heart-beats with a slight increase in their amplitude. This action takes some time before it manifests itself.

On the mammalian heart *in situ* (rabbits and cats) a slight stimulation of both the auricles and the ventricles is produced with such doses as 10 mg to 15 mg given intravenously. A slight depressant effect is observed on the isolated hearts in concentrations ranging from 1 in 30,000 to 1 in 50,000.

Central nervous system—The action of anhydrocotarnine-resorcinol-hydrochloride on the nervous system closely resembles that of narcotine, the hypnotic and sedative effects being slight. Like that alkaloid this compound appears to depress the algæsic areas in man, the sensibility to pain being decidedly decreased. Malarial patients to whom the drug was administered felt more comfortable and showed a tendency to drowsiness and sleep.

Genito-urinary system—The virgin uterus of a cat *in situ* shows a slight stimulation after an intravenous injection of 10 mg to 15 mg of the compound. The rhythmic contractions of the isolated virgin uterus of a cat, however, do not show any appreciable changes in concentrations up to 1 in 50,000. Cotarnine-hydrochloride in similar concentrations has a more powerful stimulant effect on the uterus. The automatic movements of the bladder *in situ* are slightly inhibited in doses of 10 mg to 15 mg intravenously.

Voluntary muscles—The drug appears to have no effect on the voluntary muscle in cold-blooded animals. The contractions of the gastrocnemius of the frog in a muscle-trough showed no changes when stimulated with make and break shocks from an induction coil.

Toxicity—The compound is only slightly toxic. The minimum lethal dose in the frog by injection into the dorsal lymph sac is 2 g per kilo body-weight. The lethal dose in the cat varies between 1.5 g to 2.0 g per kilo body-weight.

Chemotherapeutic studies on monkey malaria

The study of the effect of drugs on malarial parasites has hitherto been attended with many difficulties in this country. The drugs with unknown action cannot be tried in human patients and workers in the West resorted to trials on bird malaria. Here again difficulties are encountered. Firstly, the *hamoproteus* of birds cannot be compared with the malarial parasites of man, and secondly, canaries are very expensive and it is difficult to keep sparrows alive in captivity. With a view to obviate these difficulties and to obtain a suitable laboratory animal for experimentation, attempts have been made at the School for some years past to produce malaria in a suitable experimental animal. In July 1931, one of us (H G M C) found an extremely scanty infection of a plasmodium in a *Circopithecus pygerythrus* (now designated as *Macaca ira*) monkey from Singapore which resembled in certain respects the *Plasmodium vivax* parasite found in man. This infection can be communicated to the common Indian monkey, *Macacus ches* (now known as

Macaca mulatta), and Knowles and Das Gupta (1932) have made extensive studies in this connection. This parasite, although it produces a mild type of disease in *Cercopithecus pygerythrus* (*M. ira*), produces a virulent type of infection in *Macacus rhesus* (*M. mulatta*) which behaves to anti-malarial remedies, such as quinine, in much the same way as the parasites found in man. A suitable means of testing the chemotherapeutic effects of anti-malarial drugs has thus been made available.

The anti-malarial properties of anhydrocotarnine-resorcinol-hydrochloride were thus studied, *Macacus rhesus* (*M. mulatta*) infected with malaria being used for the first time for this purpose. The incubation period in this animal varies from 5 to 12 days from the date of inoculation according to the severity of the infection. Blood smears were examined every day and as soon as rings and schizonts were observed in the peripheral blood, the drug was administered in doses ranging from 3 to 5 grains. The axillary temperature was also recorded to note if the drug has got any anti-pyretic property. The following is the detail of these experiments —

Monkey I — 6th April, 1932 — A *Macacus rhesus* (*M. mulatta*) monkey was inoculated with 0.3 cc of blood from an infected *Cercopithecus pygerythrus* (*M. ira*). 7th April, 1932 to 13th April, 1932 — No parasites seen in blood film. 14th April, 1932 — The monkey looked ill, refused plantains and other food, temperature 104.5°F, blood film contained rings and schizonts, 3 to 4 in each field. 15th April, 1932 — The monkey looked very pale and restless, refused food, blood film showed heavy infection with rings, growing trophozoites and schizonts, 3 grains of the compound dissolved in water were injected in the muscles of the buttock. 16th April, 1932 — The monkey looked very ill, pallor of the face marked, blood film showed 50 to 70 parasites per field, a second dose of 3 grains was given intramuscularly but the animal died. No hæmoglobinuria was observed. Post mortem — The liver and the spleen slightly enlarged and very congested, bladder empty and contracted, other organs normal.

Monkey II — A *Macacus rhesus* (*M. mulatta*) monkey was inoculated with infected blood from a *Cercopithecus pygerythrus* (*M. ira*) on 6th April, 1932. 6th April, 1932 to 16th April, 1932 — No parasites seen in peripheral blood. 17th April, 1932 — One schizont in each field. 18th April, 1932 — 4 grains of anhydrocotarnine resorcinol hydrochloride were given intramuscularly, but the parasites went on increasing. 19th April, 1932 — 5 grains of the compound were given again but the parasites increased to 70–100 per field, pale porter colour urine was passed in the afternoon. 20th April, 1932 — The animal died during the night.

Monkey III — A *Macacus rhesus* (*M. mulatta*) inoculated with 2 drops of blood in citrated saline from monkey II just before death. 20th April, 1932 to 26th April, 1932 — No parasites seen. 27th April, 1932 — 2 growing trophozoites in each field. 28th April, 1932 — Examination of the blood showed rings and growing trophozoites and schizonts in fair numbers. 4 grains of the compound were given intramuscularly. 29th April, 1932 — 4 grains more of the drug injected but the parasites increased rapidly to 266,000 per cm as counted against red blood cells. 30th April, 1932 — An injection of quinine bihydrochloride was given in the morning and this produced a remarkable decrease in the number of parasites in the blood examined in the afternoon. The animal, however, died during the night. Post mortem — Liver and spleen congested. Hæmorrhagic patches in the peritoneum and pleura.

Monkey IV — A *Macacus rhesus* (*M. mulatta*) inoculated with blood from monkey III on 30th April, 1932. 30th April, 1932 to 7th May, 1932 — No parasites seen. 8th May, 1932 — 2–4 rings in each field and a few schizonts. Intramuscular injection of 4 grains of the compound given. 9th May, 1932 — Blood showed parasites multiplying. A further intramuscular injection of 4 grains was given but the monkey died during night of 9th May, 1932. No hæmoglobinuria occurred. Post mortem — Liver and spleen congested. Hæmorrhagic patches in the peritoneum.

Monkey V — A *Macacus rhesus* (*M. mulatta*) inoculated with blood from monkey IV on 10th May, 1932. 16th May, 1932 to 18th May, 1932 — No parasites seen. 16th May, 1932 — One schizont seen in every field. Monkey looking well and taking food well. 17th May, 1932 — Parasites found in the blood film, maturing forms fairly numerous. 5 grains of the compound were given intramuscularly. 18th May, 1932 — Parasites increased rapidly, 200,000 parasites (maturing forms) being present per c cm of blood. The animal looked very pale, anæmic. Died at 4 p.m. on 18th May, 1932.

It is evident from the above that anhydrocotarnine-resorcinol-hydrochloride has no effect in monkey malaria. The growth of parasites remained unhindered and all the animals died. Quinine in smaller doses administered at about the same time in the course of the disease stops the multiplication of parasites and the animals usually recover. The temperature, both axillary and rectal, showed a slight diminution in the monkeys following the injection of the drug but this was not a constant feature. In these animals the heat-regulating mechanism does not appear to be as well developed as in man and changes in temperature are observed with even mild excitements. Any estimate of the anti-pyretic effect of a drug from a variation in the temperature of a monkey is therefore likely to be fallacious.

Human malaria

The drug was also tried in 3 patients suffering from malaria in the Carmichael Hospital for Tropical Diseases. As it produced no effect whatsoever either on the parasites in the blood or the symptoms of the disease, further trials were considered unnecessary. The details of these cases are given below—

Case I—The patient was admitted with history of fever with rigors. Blood film showed malignant tertian rings and the temperature rose to 102°F during the course of the day. A parasite count showed 24,400 malignant tertian rings per c mm. of blood and 1,200 crescents. Anhydrocotarnine-resorcinol hydrochloride in three grain doses was administered for three days and on the fourth day five grains were given twice daily but the parasites, both sexual and asexual forms, still persisted. On the sixth day quinine sulphate was given in ten grain doses twice daily. On the seventh day the temperature came down to normal and sexual parasites completely disappeared from the blood, though the crescents persisted in smaller numbers.

Case II—A patient suffering from malignant tertian malaria showed a large number of parasites in the blood. The patient was given anhydrocotarnine-resorcinol hydrochloride in five grain doses thrice daily for five days, blood being examined daily. The temperature came down to 101°F after the first powder but varied between 101°F to 102°F during the five days that he was under treatment. The parasite persisted in the peripheral blood in increasing numbers. The patient was then put on 10 grains of quinine twice daily. The temperature subsided the next day and the peripheral blood showed no parasites on the third day.

Case III—This patient showed a mixed infection with benign tertian and malignant tertian parasites. He was at once put on to anhydrocotarnine-resorcinol hydrochloride, five grains three times a day for four days. On the third day a thick film still showed the presence of parasites. At the end of the fourth day, the parasites still persisted. The patient was then put on plasmoquine compound tablets. The parasites decreased in numbers and the patient's condition improved. The blood was free from parasites in four days and the patient made a recovery.

SUMMARY AND CONCLUSIONS

Anhydrocotarnine-resorcinol-hydrochloride is a dull white crystalline powder sparingly soluble in water. It is readily absorbed from the site of injection, after intramuscular injection no marked local reaction is produced.

The pharmacological action and toxicity of the compound have been worked out. *Paramœcium caudatum* is killed in very much the same concentrations as quinine. It has a slight depressant action on the heart and circulation, the respiration in experimental animals is stimulated. The plain muscle of the intestines and bladder is relaxed. The stimulating action on the uterine contractions is much weaker than cotarnine. Like narcotine it depresses the algæic areas of the brain.

This compound has been tried in *Macacus rhesus* (*M. mulatta*) monkeys infected with 'monkey malaria'. It has no effect whatever either on the growth of parasites or symptoms produced by the disease.

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In three human patients suffering from malaria, anhydrocotarnine-resorcinol-hydrochloride failed to produce any amelioration of the symptoms or diminution in the number of parasites in the peripheral blood. It appears to have no anti-malarial action whatever.

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THE PHARMACOLOGICAL ACTION OF AN ALKALOID OBTAINED FROM *RAUWOLFIA SERPENTINA*, BENTH

A PRELIMINARY NOTE

BY

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RAUWOLFIA SERPENTINA (Synonym—*Ophioxylon serpentinum*, Linn Roxb),
N O *Apocyanaceæ*, is a small erect glabrous shrub about one and half to three
feet in height, bearing white or pinkish flowers. It generally thrives in a rich
soil. It is known by the name of 'sarpagandha' in Sanskrit, 'chota-chand' in
Hindi, 'chandra' or 'chota-chand' in Bengali, 'dhan baiua' or 'dhan marua'
in Bihari, 'chandra', 'chota-chand', 'karavi' or 'harkai' in Bombay, 'harkaya'
in Marhatti, 'atalagandhi' or 'patala garuda' in Telugu, 'chuvana-avilpori'
in Malay. It grows quite abundantly in the tropical Himalayas along the foot of
the hills from Moradabad to Sikkim. In Assam, Pegu and Tenasserim it is found
at an altitude of about 4,000 feet. *Rauwolfia serpentina* is also found to grow wild
along the Eastern and Western Ghat ranges. In some of the northern districts of
Bihar and in Patna and Bhagalpur, it is found in abundance and is popularly known
in the bazaar as 'pagla-ka-dawa' (insanity herb).

Medicinal use—The roots, the leaves and the juice have been considered of
medicinal importance from the very early times and have attracted the attention
of the Indian and Malayan physicians. Horsefield (*Asiatic Jour*, 8) thought that
the infusion of the root is of considerable therapeutic potency. It is employed as

an anthelmintic in Java. According to Raumphius, the juice of the leaves was extensively used in India and Java as a remedy for opacity of cornea. The root was also used in case of bites of poisonous snakes and insects, both internally in the form of infusion and decoction and externally in the form of a paste. Dymock states that most of the people of the labouring classes who come to Bombay from southern Concan know the beneficial effects produced by the root in all forms of bowel complaints. It has frequently been prescribed in diarrhoea and dysentery. In cases of cholera the root is combined with the root of *Aristolochia indica*, in colic and dysentery, one part of *Rauwolfia serpentina* root with two parts of *Holarhena antidysenterica* root and three parts of *Jatropha curcas* are given in milk, and if there is fever it is given in combination with the root of *Andrographis*, ginger and black salt. The dose in each case is from 3 to 4 tolas. It was also supposed to increase the contractions of the uterus during childbirth and earned a reputation as an ecbolic. The interest in the drug has recently been stimulated on account of its well-marked hypnotic and sedative properties. No mention of these properties occurs in the old literature on Indian medicinal plants. The hypnotic and sedative action of the drug, however, appears to have been known to the poorer classes in Bihar and the practice of putting children to sleep by this drug is stated to be still present in certain parts of that province.

Chemistry of Rauwolfia—The investigation into the chemical composition of the root has been carried out by a number of workers. Bettink (1888) carried out an analysis of the root and found a crystalline body related to juglone but no alkaloid. Later Erykman discovered an alkaloid in the root of an Indian species of the plant. Greshoff (1890) isolated a principle which gave all the reactions of an alkaloid. Dymock (1895) carried out an elaborate investigation into the chemical nature of the root and detected the presence of an alkaloid. Recently the root has been re-examined by a number of workers. Sen and Bose (1931) have found two alkaloids in the root with different melting points. The quantity of the total alkaloids has been estimated to be fairly high, amounting to about one per cent of the dried roots. The root also contains a lot of resin and starch and, when incinerated, leaves about eight per cent of ash consisting mainly of potassium carbonate, phosphate, silicate and traces of iron and manganese.

Siddiqui and Siddiqui (1931) have found five new alkaloids to which they have given special names, as follows—

GROUP A—Ajmaline group, consists of three white crystalline weak bases

	Melting point, Centigrade	Percentage
1 Ajmaline	158°–160°	0.1
2 Ajmalinine	180°–181°	0.05
3 Ajmalicine	250°–252°	0.02

GROUP B—Serpentine group—two bright yellow crystalline stronger bases

	Melting point, (no grade	Percentage
1 Serpentine	133°-151°	0.08
2 Serpentinine	163°-165°	(decomposes) 0.08

Other constituents identified are (a) a phytosterol, (b) oleic acid, and (c) unsaturated alcohols of formula $C_{25}H_{44}O_2$

van Itallie and Steenhauer (1932) mention the presence of at least three alkaloids, the nature and identity of these being more or less the same as found by Siddiqui and Siddiqui. The other constituents are a fatty oil, a phytosterol, glucose, sucrose, fumaric acid, a compound similar in nature to an oxymethylantraquinone derivative and a strongly fluorescent substance.

As a result of the work carried out in the Department of Chemistry, School of Tropical Medicine, Calcutta, an alkaloidal base has been isolated from the root in a pure condition. The process of extraction of the alkaloid was carried out as follows. The powdered root was extracted with rectified spirit, the alcohol distilled off, the residue extracted with hot acidulated (HCl) water till the alkaloid was completely removed. The acidulated solution was cooled with ice and treated with petroleum ether for the removal of oily substances. The solution was then neutralized with caustic soda, filtered and then taken up with chloroform. The chloroform extract was then concentrated and acidulated with dilute hydrochloric acid. From this solution the total alkaloids present were precipitated with dilute caustic soda, washed and dried. The total base was further purified for pharmacological experiments. A quantity of an alkaloid hydrochloride was isolated from the total base and it corresponded to the one described by Siddiqui as ajmaline hydrochloride. The hydrochloride crystallized in boat-shaped needles and melted at 135°C. It is soluble in hot water but only slightly in cold and has a bitter taste.

More recently Siddiqui and Siddiqui (1932) have revised their work on some of the alkaloids isolated by them and have suggested some modifications in their formulæ.

EXPERIMENTAL

The alkaloid used in our experiment was isolated in our laboratory. It was a dull brownish-yellow crystalline substance, sparingly soluble in water. A one per cent solution was used in the majority of our experiments. This formed a clear, pale straw-yellow solution which did not show any change on keeping for three to four days in the ordinary room temperature.

Action on Paramœcium caudatum —The effect of the alkaloid has been studied on lower forms of life like *Paramœcium caudatum*. Table I shows the effect of the different dilutions of the alkaloid —

TABLE I

Action of alkaloid of R. serpentina on P. caudatum

Dilution of alkaloid used	Movement becomes sluggish Time	Death Time
1 in 200	At once	At once
1 in 500	"	$\frac{1}{2}$ minute
1 in 1,000	$\frac{1}{2}$ minute	1 "
1 in 4,000	2 minutes	4 minutes
1 in 8,000	3 $\frac{1}{2}$ "	6 "
1 in 10,000	8 "	20 " (60 per cent)
		25 " (all)
1 in 20,000	10 "	23 " (40 per cent)
		1 hour, (all)
1 in 40,000	40 "	1 hour 20 minutes
		(40 per cent)
1 in 50,000	No effect	No effect in 4 $\frac{1}{2}$ hours

A perusal of the above table will show that the alkaloid is definitely toxic in a dilution up to 1 in 20,000.

Toxicity —Numerous experiments were performed on different species of animals to determine the lethality and the maximum tolerated dose. The results are given in Table II.

It will be seen from Table II that the toxicity of the alkaloid varies within wide limits in different animals. Very large doses are tolerated by the frogs (*Bufo melanostictus*), while the lethal dose for white mice (Kasauli strain) is comparatively low both by the intraperitoneal and the intravenous route. A hypodermic dose of 0.15 g (150 mg) is perfectly tolerated by the guinea-pigs and cats, while an intravenous or intraperitoneal injection of less than half the quantity is found to be definitely lethal. The unusual difference in activity of intraperitoneal and intravenous as compared with subcutaneous injections might be explained by an unusually slow absorption from the subcutaneous tissues. A dose of 25 mg to 30 mg per kilo body-weight is tolerated whether given intraperitoneally or intravenously in warm-blooded animals and should therefore be looked upon as the maximum tolerated dose.

TABLE II
Mean toxicity of alkaloid of R. serpentina

Average weights —	Frogs, 25 g		White mice, 20 g		Guinea pigs, 350 g		Cats, 2,000 g		
	Subcutaneous injection (mean of 6 observations)	Lymph sac (mean of 15 observations)	Intraperitoneal injection (mean of 10 observations)	Lal vein (mean of 10 observations)	Subcutaneous injection (mean of 3 observations)	Intraperitoneal injection (mean of 10 observations)	Subcutaneous injection (mean of 3 observations)	Intraperitoneal injection (mean of 10 observations)	Intravenous injection (mean of 6 observations)
Route —									
Mean dose —	1.6 mg to 2 mg per g body weight	1.1 mg to 1.5 mg per g body weight	0.1 mg to 0.12 mg per g body weight	0.05 mg to 0.12 mg per g body weight	150 mg per kilo body weight	75 mg per kilo body weight	150 mg per kilo body weight The animal did not die for two days. Lethal dose not determined for want of alkaloid	80 mg per kilo body weight	25 mg per kilo body weight

Local effects —One per cent solution of the alkaloid instilled into the eyes of a rabbit does not produce anaesthesia of cornea nor has it any effect on the pupil. Subcutaneous and intramuscular injections of one to two per cent solution do not give rise to any signs of local irritation.

Alimentary system —The effect of the alkaloid on the intestines *in situ* was studied by Jackson's enterograph on cats under chloralose anaesthesia. After an injection of 5 mg. of the alkaloid into the femoral vein there is a distinct stimulation of both the tonus and the peristaltic movements, a summation or additive effect is sometimes obtained when another injection is given on the top of the first injection. With 10 mg. the movements become much more marked (Graph II, fig 10). This effect is still elicited if the vagal nerve-endings are paralysed with atropine, showing that the vagus does not play any active part in the production of the effect.

The effect of the alkaloid on isolated pieces of cat's intestine was studied in Dale's uterine bath, oxygenated Fleisch's solution at pH 7.4 and temperature 37.5°C being used for perfusion. In dilutions of 1-50,000 to 1-25,000, the tone of the musculature as well as the pendulum movements are definitely increased (Graph II, fig 8). This increase in the movements is fairly steady and continuous and is generally maintained from 10 to 15 minutes.

Respiratory system —After 0.5 mg. of the alkaloid is given intravenously, little if any change is produced in the respiratory movements, though sometimes slight initial stimulation is observed. With larger doses, i.e., 5 mg. to 10 mg. and upwards, there is slowing of the rate as well as depth of respiration (Graph I, figs 1 and 2). This phenomenon is still observed after the vagal nerve-endings have been paralysed with atropine, and therefore it is probable that depression of the respiratory centre in the medulla plays some part in its causation. That the alkaloid has a sedative action on the respiratory centre is evident from the fact that when a small dose of the drug is injected directly into the cisterna cerebello-medullaris the animal shows signs of immediate failure of respiration while the vasomotor centre remains unaffected.

The action of the drug on the bronchioles was tested by recording the intra-pleural pressure by means of a canula introduced through the ribs into the pleural cavity. The animals were kept under artificial respiration from a mechanical pump with regulated number of strokes per minute, so arranged that the quantity of air pumped into the lungs remained quite constant. The alkaloid of *R. serpentina* in 5 mg. doses does not produce any appreciable change in the intra-pleural pressure. With toxic doses certain changes are observed which are probably due to deficient air entry into the lungs on account of the depression of the respiratory centre.

Circulatory system *Effect on systemic blood-pressure* —Injection of 5 mg. of the alkaloid into the femoral vein produces, in the majority of animals, a fall of the carotid blood-pressure, varying from 10 to 15 mm. of Hg, within five seconds of administration. This fall in pressure is maintained for a considerable time and the blood-pressure rarely resumes its former level. With increasing dosage, e.g., 7.5 mg. to 10 mg., this fall in pressure becomes more marked and there is distinct slowing of the heart-beats (Graph I, figs 2 and 5, and Graph II, figs 7 and 10). When

large doses are injected the fall in pressure may amount to 50 mm of mercury before collapse occurs and death supervenes. Rarely this fall in blood-pressure may be slight and even a small initial rise lasting from 5 to 10 minutes has been observed (Graph I, fig 1). In decerebrated cats, in which the medulla and the higher parts of the brain have been destroyed through the foramen magnum, the fall in blood-pressure is much less evident. The fall in blood-pressure is also observed after the terminations of the vagi have been paralysed with atropine, showing that vagal inhibition does not play much part in the fall produced.

Effect on the heart. Amphibian heart—The alkaloid produces a distinct slowing of the heart-rate after it is injected in the lymph sac or applied directly to the heart. After continued application the systole becomes progressively weaker, diastole is prolonged, the heart becomes gradually dilated and finally stops in diastole.

Mammalian heart. Myocardiograph experiments—With 5 mg doses, there is slight diminution in the amplitude of the auricular and the ventricular systole (Graph I, fig 1). With larger doses, e.g., 10 mg to 15 mg, a distinct slowing of the rhythm is evident and the auricles and the ventricles become markedly depressed, the effect on the auricles being more marked (Graph I, fig 3). Gradually some inco-ordination in the passage of the impulses from the sino-auricular node to the auriculo-ventricular node becomes evident and the beats become irregular. With larger doses, the amplitude of auricular contractions is greatly diminished and there is rapid weakening in strength leading to fibrillary twitchings of the auricle and ultimate paralysis. This effect is not abolished if the vagi are severed in the neck or if the nerve-endings are paralysed with atropine (Graph I, fig 3). In decerebrated animals very similar effects are produced. These experiments show that neither the cardio-inhibitory centre nor the vagi are responsible for the effect produced. The sympathetics also do not appear to play any part as paralysis of the sympathetic endings with large doses of ergotoxine does not alter the myocardiographic tracing in any way. It is therefore probable that the depression of the cardiac musculature is responsible for the slowing of the cardiac rhythm.

Cardiometer experiments—The changes in the volume of the heart under the effect of this alkaloid were studied by means of a glass cardiometer. An injection of 5 mg produced diminution of both the systolic and diastolic phases of the heart, the cardiac output was diminished and the heart as a whole appeared to be depressed (Graph I, fig 5). After giving 8 mg of atropine intravenously, so as to paralyse the vagal endings in the heart, the same effects were produced.

Action on the isolated heart—Isolated hearts of rabbits and kittens were perfused according to Langendorff's method with oxygenated Locke's solution (pH 7.2 and temperature 37.5°C) to which defibrinated blood was added. The alkaloid in concentrations of 1 in 400,000 produced a slight depression of the heart. On increasing the concentration of the solution to 1 in 200,000 to 1 in 100,000 the weakening of the force of cardiac contraction became more apparent. With still higher concentration, such as 1 in 50,000, the depression was immediate and well marked. All these effects could be elicited even after the terminations of the vagi had been paralysed with atropine and point to direct effect on the cardiac musculature.

Action on blood vessels—It has been pointed out that the blood-pressure falls after the intravenous administration of the alkaloid. This appears to be due mainly

to diminished cardiac output as is evident from the myocardiograph and perfusion experiments on the isolated heart. In the decerebrated animals the fall is less marked and therefore it is likely that dilatation of the blood vessels as a result of the depression of the vasomotor centre may be one of the factors in lowering the blood-pressure. In order to determine whether the local effect on the plain muscle of the blood vessels is also responsible for this dilatation, a Trendelenburg preparation was put up and the vessels were perfused with frog Ringer's solution to which the alkaloid was added. The number of drops of the perfusate per minute definitely decreased, showing thereby that vaso-dilatation was produced. This vaso-dilatation is due to the direct inhibitory effect of the drug on the musculature of the blood vessels. In warm-blooded animals, such as the cat, addition of the alkaloid to the perfusate produces an appreciable dilatation of the blood vessels of the artificially perfused hind limbs. The dilatation is still present, though to a much less extent, after the vasomotor nerve-endings are paralysed with ergotoxine.

Volume changes of the abdominal organs—The effect on the blood vessels of the splanchnic area was studied by noting the changes produced on the volume of such organs as the spleen, kidney and the intestines. The intestinal volume, spleen volume and kidney volume all show a slight increase in the majority of experiments. This is probably secondary to the fall in systemic pressure and consequent splanchnic congestion (Graph II, figs 6 and 7).

Limb volume—There is little or no change in the limb volume if the alkaloid is given in doses of 5 mg. Sometimes a decrease in the volume is observed which can possibly be accounted for by the local depletion of blood caused by accumulation in the splanchnic area (Graph I, fig 2).

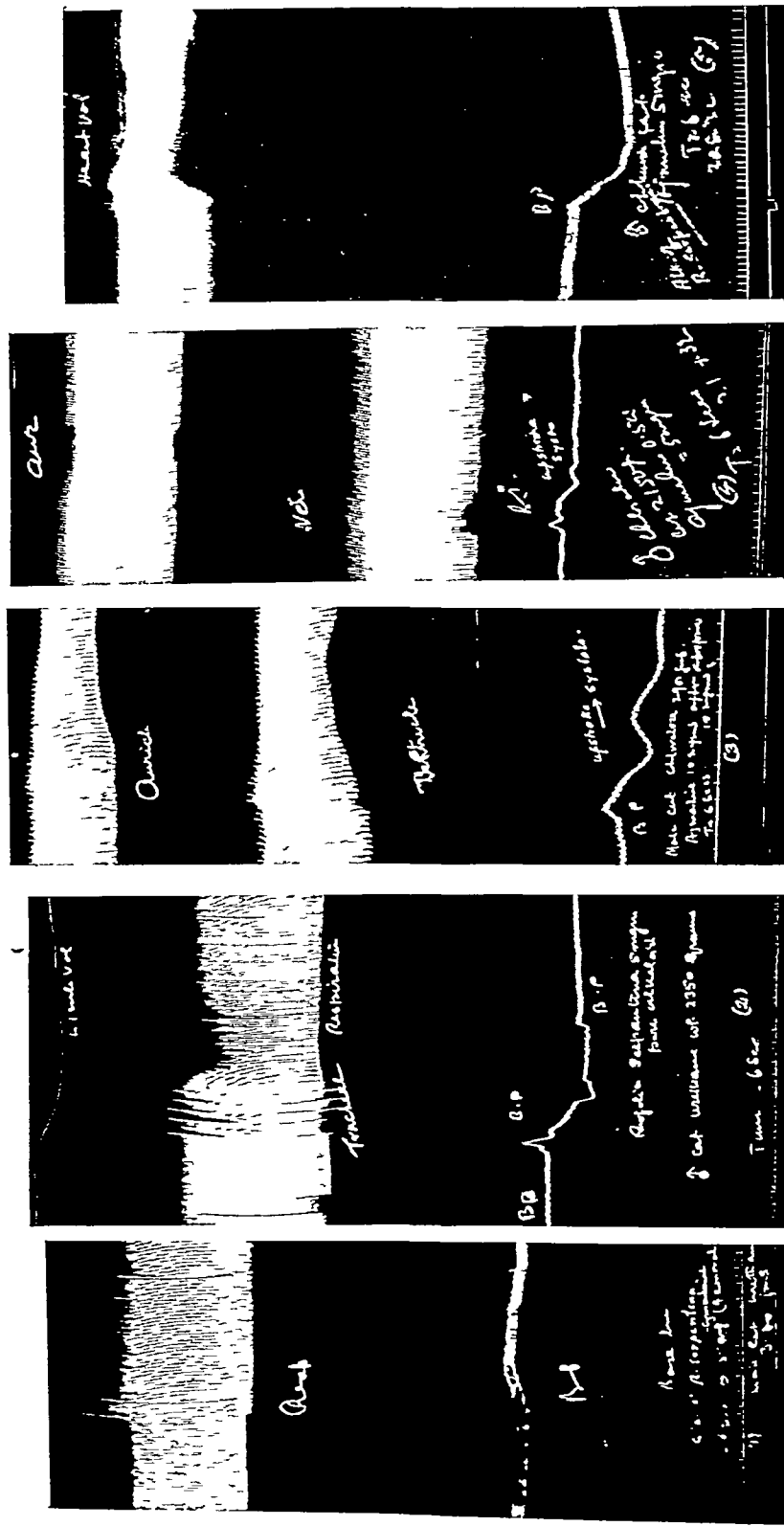
Action on the uterus—The alkaloid in doses of 5 mg. to 10 mg. intravenously has a definite stimulating action on the tone and rhythmic movements of the virgin cat's uterus *in situ* (Graph II, fig 11). Pregnant uterus is also stimulated. Isolated strips of uterus from rabbits and cats, when perfused in Dale's bath, also show stimulation in concentration varying from 1 in 100,000 to 1 in 50,000 (Graph II, fig 9).

Action on the central nervous system—As *Rauwolfia serpentina* has earned a reputation as a sedative and hypnotic the effects of the alkaloid on the central nervous system were investigated in more detail.

Cold-blooded animals. Fishes—The hypnotic effect on fishes was studied by Overton's method. Three 'koi' fishes (*Anabas scandens*?) were placed in a glass-jar containing 200 c.c. to 300 c.c. of a one per cent solution of the alkaloid. In a similar jar filled with water three similar fishes were kept under the same conditions to serve as control. The fishes after immersion did not behave in a manner indicating that the solution had any irritant effects. They were touched with a glass-rod every minute for the first twenty minutes and then at longer intervals. The fishes showed a slight but distinct inhibition of spontaneous motion. Upon removal to fresh water, the fishes recovered rapidly and regained their normal condition.

Frogs (Bufo)—Injections of the alkaloid were given into the anterior lymph sac of frogs weighing about 50 g. Doses of less than 0.1 g. produced no effect whatever. With increasing doses a series of symptoms were observed which were quite characteristic. Within 5 to 7 minutes of the injection these amphibia show a

GRAPH I



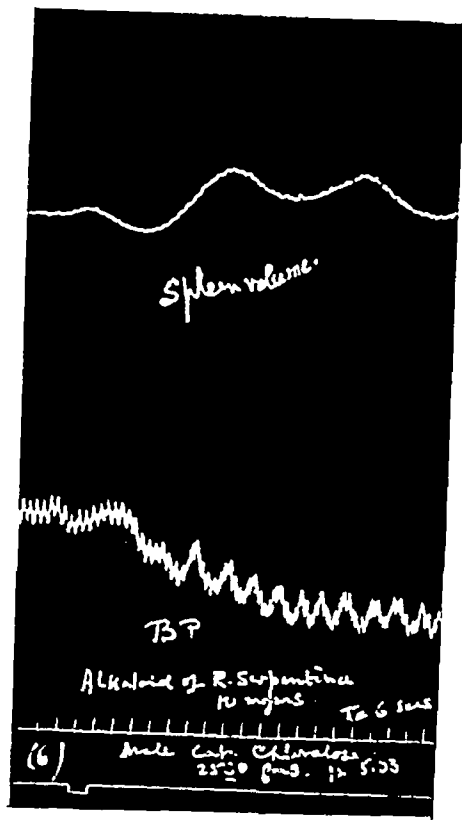


Fig 6.

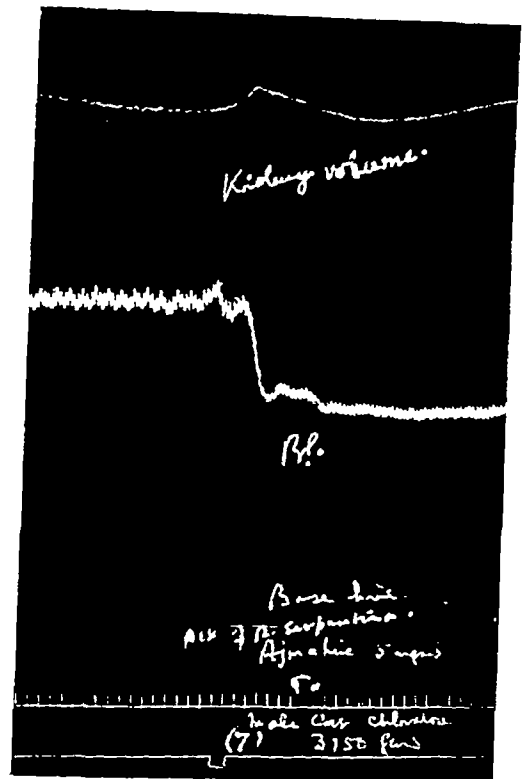


Fig 7

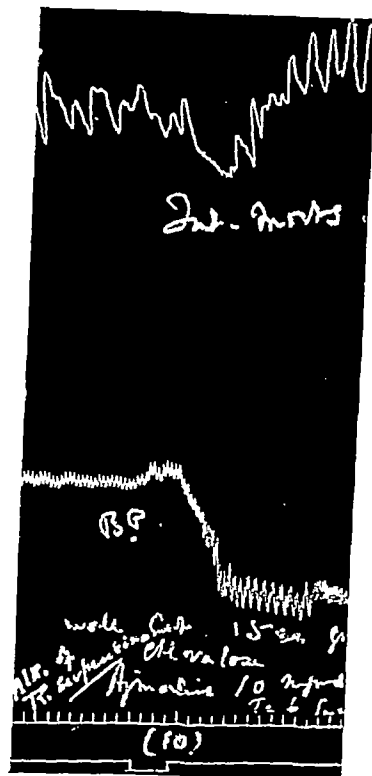


Fig 10

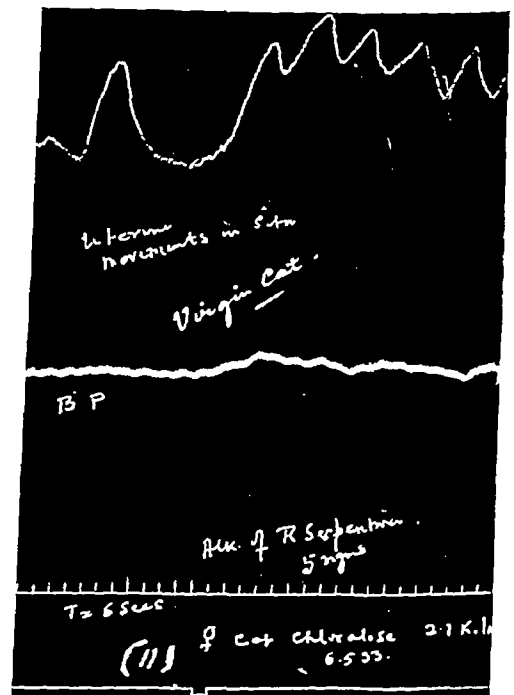


Fig 11

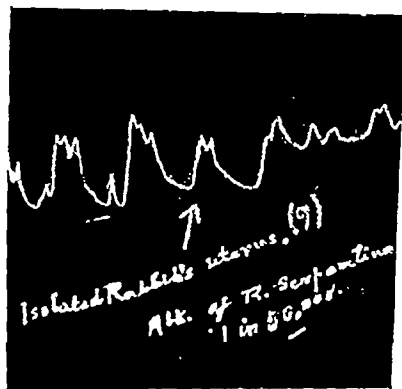


Fig 9

- Figs 6 and 7 Spleen volume and kidney volume Note fall in pressure and rise in the volume of both the organs
 Fig 8 Isolated loop of cat's intestine Note stimulation of intestinal movements in dilution of 1 in 100,000
 " 9 Isolated uterus in Dale's bath Stimulation of both tone and pendulum movements in dilution of 1 in 50,000
 " 10. Blood pressure and intestinal movements *in situ* Note fall in blood pressure and increase in the tone and movements of the intestines
 " 11. Blood pressure and uterine movements *in situ* Note increased tone of the uterine contractions

tendency to inhibition of voluntary movements which is in marked contrast to the brisk movements of the controls. The frogs react normally when stimulated at this stage, but gradually the movements become sluggish. They sit quietly in their usual posture but their leaps become clumsy and less brisk. Sensory conduction appears to be definitely impaired at this stage as a mild prick with a needle or pinching with a pair of forceps usually elicits no reflex response. Chemical stimuli to the skin, e.g., moistening the skin with acetic acid and stimulation of the viscera, which produce defensive movements in normal frogs, are without effect. The depression deepens and in 15 minutes or so, the frog if turned on its back does not attempt to right itself. The limbs are markedly relaxed, giving an appearance resembling that of a pithed frog. The perception of pain-stimuli appears to be decidedly decreased. Electrical stimulation by tetanizing current fails to produce any effect and the toes can be clipped with scissors without any reflex motor response. The heart-beats become progressively slower from the beginning but the organ goes on beating for over an hour.

Warm-blooded animals *White mice*, weighing on an average 20 mg, were given a two per cent solution of the alkaloid intraperitoneally in sublethal doses. With doses ranging from 0.27 mg to 0.4 mg per g body-weight, there is initial stimulation as shown by increased excitability and hurried respiration. This picture, however, soon changes and the animal assumes a quiet and crouching attitude and prefers to remain undisturbed. The receptivity to external stimuli e.g., pricks with needles or the electric current, remains intact. Larger doses (i.e., those below the lethal limit) produce loss of sensation and sometimes actual narcosis. Death may occur later from failure of respiration.

Guinea-pigs—Three guinea-pigs were given the alkaloid in doses of 50 mg, 75 mg and 100 mg per kilo body-weight subcutaneously. Within 20 to 30 minutes the animals receiving 75 mg and 100 mg per kilo showed a tendency to lie on one side and became listless and apathetic. Perception to touch and pain-stimuli, however, remained unaltered. The effect did not last for more than one and a half hours and passed off gradually.

Intraperitoneal injections of the same dosage gave a characteristic train of symptoms quite unlike those observed with subcutaneous injection. With 100 mg dosage, the guinea-pig developed within 5 to 10 minutes nodding movements of the head. In another 10 minutes the animal was seen to lose its balance and lay on its side, breathing heavily. Within 30 minutes, it showed signs of collapse, just before death, twitchings of isolated group of muscles and convulsive seizures were observed. The tonic contractions lasted for a few seconds and were followed by complete relaxation. The convulsions recurred at intervals till death supervened from failure of respiration. The animal receiving 75 mg per kilo showed drowsiness and appeared to go into a deep sleep in 15 to 20 minutes, but this effect passed off in $1\frac{1}{2}$ hours.

Cats—Two series of three cats, each weighing on an average from two to three kilos, were given injections of the alkaloid subcutaneously in doses varying from 50 mg to 100 mg per kilo body-weight. Excepting a temporary drowsiness and disinclination to move about, no other effects pointing to a depression of the higher centres were observed. This lasted only for a short time

Intravenous injections in the saphenous vein or intraperitoneal injections of 50 mg per kilo produce within 5 to 8 minutes salivation and a tendency to vomit. The animal looked wild and had dilated pupils. The head rolled from side to side and soon the animal lost its equilibrium and lay on one side. The respiration became laboured and consciousness was lost. Sensations were still slightly maintained though dulled considerably.

Cerebral centres —With a view to determine whether the drug had any special action on the cerebral centres a small quantity (10 mg) of the drug was injected directly into the cisterna cerebello-medullaris through a needle introduced through the occipito-atlantal ligament. Only light ether anaesthesia was used so that the ether effect could pass off within a short time. When these animals recovered consciousness the alkaloid was injected and the effects produced were carefully observed. Within 40 to 60 seconds following the injection, drowsiness and somnolence were observed and the animal became narcotized. During this period the reflexes generally, and the corneal reflex in particular, were lost. Sensory and motor conduction were, however, interfered with only very slightly. Application of stimuli from a secondary coil produced local reflexes but these appeared to show a diminished response when compared with those of a normal animal. If at this stage a solution of strychnine (2 mg) is introduced the animal develops the typical convulsions but the seizures are distinctly less powerful. These convulsive movements may be decreased again by injecting another dose of the alkaloid thus showing that the excitability is markedly depressed by the alkaloid.

Anti-pyretic effect Action on the heat-regulating centre —The action on the heat-regulating centre was determined by producing experimental pyrexia in rabbits by Kilhone's method. This consists in injecting hypodermically a culture of *B. coli communis* grown in broth for four days and then killed by heating to 65°C for one hour. Daily record of the rectal temperature was kept at four-hourly intervals for three days to get a mean record of the normal temperature, great care being taken to handle the rabbits gently to avoid excitement. The drug was then injected subcutaneously and intramuscularly and half-hourly temperatures were recorded. It was found that the drug in doses of 50 mg per kilo reduced the temperature to 1.5 to 2 degrees Fahrenheit and the temperature was usually maintained at this level. The alkaloid thus has anti-pyretic properties resembling the coal-tar group of drugs.

Sensory nerve fibres —In order to ascertain whether the alkaloid exerts its depressant action through the sensory nerve fibres cats under urethane anaesthesia were used. The sciatic nerve in the leg was exposed and after the minimal current producing a definite change in the respiration had been determined, a two per cent solution of the alkaloid was dropped on to the segment of the nerve, and the latter was stimulated both below and above the point of application with the same strength of current. It was found that in none of the animals was the alkaloid able to block the sensory impulses from going to the central nervous system, while cocaine crystals similarly applied to the same nerves uniformly paralysed the point of application within five minutes. The diminished response on stimulation of the local reflexes following the administration of the drug is therefore possibly due to the depression of the nerve-cells of the reflex arc.

SUMMARY AND CONCLUSIONS

(1) *Rauwolfia serpentina* is reputed as a cure for insanity, epilepsy, high blood-pressure, etc

(2) A crystalline alkaloid having a melting point of 135°C was isolated and its pharmacological action worked out. It is sparingly soluble in cold water but is more soluble in hot water.

(3) The alkaloid is toxic to *Paramœcium caudatum* in concentrations of 1 in 20,000 and more. Its toxicity varies with different species of animals. The lethal doses for frogs, white mice, guinea-pigs and cats have been determined.

(4) The alkaloid has a stimulant effect on the plain muscle of the intestine and the uterus.

(5) The systemic blood-pressure falls due to dilatation of the blood vessels of the splanchnic area. The respiration is depressed, death occurring from failure of respiration due to the paralysing effect of the alkaloid on the respiratory centre.

(6) The alkaloid has a pronounced effect on the central nervous system. In sublethal doses injected into the lymph sac of frogs narcosis quickly ensues. In mammals the alkaloid produces symptoms which are attributable to its depressing effect on various cerebral centres in the reverse order of their development. The short period of excitement seen in guinea-pigs and cats is probably due to the dissolution of the higher centres as is so often seen with morphine, chloroform and alcohol. There is also evidence to show that there is some depression of all nerve-cells in the body.

(7) The alkaloid on account of its cerebral depressant properties should prove to be a valuable sedative drug. Its depressant effect on the respiratory centre should, however, be borne in mind. It lowers the blood-pressure and if administered in proper dosage should be of value as a remedy against hyperpiesis. Purgation is usually produced by the drug on account of its stimulant properties on the plain muscles of the gastro-intestinal tract. Its stimulant effect on the uterine movements, both virgin and pregnant, coupled with its pain-relieving properties should be useful during parturition. The drug is likely to be a valuable addition to the armamentarium of physicians and further work to place it on a definite therapeutic basis is in progress.

We are grateful to Dr S Ganguly for his help in connection with this work.

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ON THE ELECTRIC CHARGE OF ERYTHROCYTES

Part II

MALARIA

BY

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IN a previous paper (Chopra and Chaudhury, 1932) we studied the effect of quinine bihydrochloride on the negative charge of normal red blood corpuscles at different pH values. The results indicated that the charge of the erythrocytes is affected by very low concentrations of the alkaloid, though the diminution in charge is very small. It was then thought desirable to study the charge of erythrocytes obtained from patients suffering from malaria and, in the present paper, some preliminary observations are given which actually show that the negative charge of the red cells obtained from malarial blood is different from that of the normal erythrocytes.

EXPERIMENTAL

The method of measurement of charge was the same as described in our previous paper (*loc cit*). With the cell used, the erythrocytes infected with malarial parasites could not be distinguished under the microscope from those not infected. One per cent suspensions of erythrocytes in normal saline obtained from malarial patients were prepared in the same way as in our

previous experiments The results expressed in cm per second per volt per cm are given in Tables I and II —

TABLE I

Showing the cataphoretic velocity of red blood cells from different normal individuals

Number	$V \times 10^5$	Number	$V \times 10^5$
1	11.1	6	11.17
2	12.7	7	12.0
3	11.3	8	12.05
4	12.5	9	11.5
5	13.1	10	12.3

Mean velocity = 12.05×10^5 cm per second per volt per cm

TABLE II

Showing the cataphoretic velocity of one per cent suspension of red blood cells obtained from malaria patients

Number	$V \times 10^5$	REMARKS
1	13.4	M T rings and crescents
2	8.3	Scanty quartan, trophozoites and gametocytes
3	12.7	B T trophozoites, schizonts and gametocytes
4	10.0	Scanty M T rings and crescents
5	14.6	M T rings
6	16.5	Quartan trophozoites and schizonts
7	14.3	M T rings

DISCUSSION

The results in Tables I and II show that comparatively there is a great variation in the charge of the red blood cells obtained from different patients suffering from malaria. The mean of the observations in Table II, however, is not of any particular significance, it would rather tend to show that these differences are ascribable to different conditions of the red blood cells of patients suffering from malaria. It might be argued that the red cells, seen moving under the microscope at different heights of the chamber, are not actually infected, but this consideration probably does not detract from the value of these results. Brown (1933) with similar technique showed that there appears to be no difference in the electric charge of cells containing parasites and the healthy cells in an infected bird. Moreover, each of the observations is a mean of at least ten different readings and in no case was there any appreciable difference in the time for the different cells of the same suspensions to pass through a definite distance.

It will be seen that in Nos. 2 and 4 there is a decrease and in Nos. 5, 6 and 7 an increase in the electrical charge of the cells. Brown (*loc cit*), however, found a decrease in the charge of the red cells obtained from malaria birds, but his medium of suspension contained serum, and to this might be attributed the divergent results obtained.

There is at present a general tendency to ascribe phagocytosis to the lowering of the charge of bacteria and cells. The lowering of the charge of the erythrocytes by quinine also favours this view, for this lowering would favour phagocytosis of the infected red cells and thus help in the cure of malaria. It is, of course, conceivable that the electrical forces of repulsion between a white cell and the red cell will be less on addition of quinine with the result that the white cells can easily engulf the infected red cells.

This simple theory of a living process is borrowed from the allied phenomenon of coagulation of colloids, where the particles acquiring lower electrical charge are thought to be aggregating into larger ones, but recently it has been shown by Kruyt and his co-workers (1926, 1927) and Mukherjee and his co-workers (1925, 1927) that colloid particles even with high charge can conglomerate. Before advancing any definite theory on the process of phagocytosis it appears to us that experiments *in vivo* on the charge of the white and the red cells and its dependence on their morphology as well as its variation with mixtures of quinine and serum, both *in vivo* and *in vitro* are necessary. Such experiments on monkey malaria are in progress and it is hoped that these results will be communicated shortly.

CONCLUSIONS

The negative charge of red cells suspended in normal saline obtained from malarial blood is different from that of normal red cells, in some cases there is a decrease and in others an increase.

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PHARMACOLOGICAL ACTION OF KURCHICINE (AN
ALKALOID OF *HOLARRHENA*
ANTIDYSENTERICA)

BY

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SINCE the publication of the work on the pharmacological action of conessine by Chopra and his collaborators (1927), a large amount of work has been done on the chemistry of the bark of *H. antidysenterica*. Ghosh and Ghosh (1928) isolated conessine from the Indian bark and discovered two new hitherto unrecognized alkaloids which they named *kurchine* and *kurchicine*. Ghosh and Bose (1932) published in detail the composition and properties of *kurchine* and *kurchicine* and their salts. Haworth (1932) isolated an alkaloid named oxy-conessine from the seeds of the same plant which was found to be identical in chemical composition with *kurchine*. Siddiqui and Siddiqui (1932) and later Bertho, Schuckmaun and Schonberger (1933) have reported the presence of other alkaloids in the bark of the Indian *Holarrhena antidysenterica*.

Messrs Whiffen and Sons, Ltd, of Aldgate Chemical Works, London, kindly sent us a quantity of a white crystalline powder isolated from *H. antidysenterica*. This was labelled as 'Kurchine' hydrochloride, on examination by the Department of Chemistry it was found to be very similar in properties to *kurchicine* described by Ghosh and Bose. The proportion of this alkaloid in the bark varies in specimens obtained from different localities, but usually it occurs in smaller quantities than conessine and the low-melting-point alkaloid *kurchine*. The hydrochloride forms well-defined crystals, is very soluble in water and is therefore suitable for oral and parenteral administration. A detailed action of this alkaloid (*kurchicine* of Ghosh

and Ghosh and labelled as kurchine by Messrs Whiffen and Son, Ltd) has been worked out and a short account of this is given in this paper

Action upon the undifferentiated protoplasm—The comparative effect of kurchine and emetine hydrochloride solutions on *Paramœcium caudatum* are given in tabular form below (Table I) —

TABLE I

Showing the behaviour of Paramœcium caudatum towards kurchine and emetine in different dilutions. The pH of the solution was kept at 7.1

Number.	Strength of solution	Effect of kurchine on <i>Paramœcium</i>	Effect of emetine on <i>Paramœcium</i>
1	1-50	Instantaneous death	Instantaneous death
2	1-100	"	"
3	1-500	Death in 10 seconds	Death in 15 seconds
4	1-1,000	" " 1 minute	" " 2 minutes
5	1-2,000	" " 2 minutes	" " 3 "
6	1-4,000	" " 4 "	" " 4 "
7	1-8,000	" " 11 "	" " 12 "
8	1-10,000	" " 12 "	" " 13 "
9	1-16,000	" " 10 "	" " 14 "
10	1-20,000	" " 1 " 13 seconds	" " 6 " 13 seconds
11	1-32,000	" " 8 "	" " 14 "
12	1-40,000	" " 11 " 15 "	" " 12 "
13	1-80,000	" " 15 "	" " 19 " 30 "
14	1-100,000	" " 23 " 30 "	" " 35 "
15	1-200,000	" " 43 " 30 "	" " 43 " 30 "
16	1-250,000	" " 15 "	" " 1 hour 15 minutes

N.B.—Controls kept under similar conditions were alive and active

A perusal of the above table will show that *kurchine* in such high dilutions as 1 in 100,000 to 1 in 250,000 is destructive to the *Paramœcium* and that its action

runs almost parallel with emetine in this respect Kurchicine like emetine is therefore a general protoplasmic poison

TOXICITY —

TABLE II

Showing results of toxicity experiments

Animals used	Route of administration	Average M L D per kilo	Number of observations	Symptoms and post mortem findings
White mice	Intravenous	38.12 mg	4	Involuntary defæcation, micturition spasms, rapid respiration, post mortem findings—congestion of lungs and kidneys, heart empty and arrested in systole
Guinea pigs	Intramuscular	64.3 „	5	Do
Cats	Oral	250.0 „	2	Hurried respiration and signs of excitement, salivation, diarrhoea greenish stools, unsteady gait, incoordinated movements and strychnine like convulsions

A study of the above results will show that kurchicine is a very toxic alkaloid

Local action—Kurchicine has no irritant effect on the skin. A 4 per cent solution of kurchicine hydrochloride when instilled into the eye of a rabbit produced partial anæsthesia of the cornea within 10 to 12 minutes. Equal concentrations of cocaine hydrochloride solution produced similar results in 4 to 5 minutes. There were no signs of irritation as evidenced by lachrymation or congestion of the corneal vessels. The partial anæsthesia thus obtained lasted for not more than three-quarters of an hour. Intramuscular injections into the gluteal muscles of a cat did not produce any marked reaction in the form of inflammation or necrosis.

Alimentary system—*Digestive enzymes* Kurchicine in high dilutions (1 in 200,000 to 1 in 100,000) increases the power of the starch-splitting enzyme Ptyalin present in the saliva. Peptic digestion does not seem to be affected even by such concentrations as 1 in 1,000 to 1 in 5,000.

Intestinal movements—Intravenous injections of 2 mg of kurchicine were given to an anæsthetized cat, the movements being recorded with Jackson's enterograph. There is slight increase in the tone of the musculature and stimulation of the peristaltic movements of the ileum. Similar results were obtained after paralysing the vagal nerve-endings with atropine, suggesting the possibility of

direct action of the drug on the intestinal musculature. On the isolated intestines of cats and rabbits in a Dale's uterine bath, 1 in 25,000 to 1 in 50,000 dilutions produce a slight stimulation of both the tone and the movements. Emetine in higher dilutions, i.e., 1 in 200,000 to 1 in 100,000 produces a marked stimulation of both the tone and peristaltic movements.

Respiratory system—Intravenous injections of 2 mg of the drug were given to cats, the respiratory movements being recorded through a canula introduced into the trachea. The respirations are slightly stimulated, probably reflexly due to the fall in blood-pressure. With large doses, e.g., 5 mg, the respiratory movements show marked stimulation at first but soon stop altogether. The heart continues beating long after the respirations have ceased.

Cardiovascular system—Blood-pressure Intravenous injection of 1 to 2 mg of kurchicine in a chloralosed cat produces, within 10 to 20 seconds, a distinct fall in blood-pressure, varying from 15 mm to 40 mm of mercury. This fall is maintained for a long time. In decerebrated animals the same fall is observed but to a much smaller extent, showing that the medullary centres probably play no part in this action. This fall is also produced after paralysing the vagal and sympathetic nerve-endings with atropine and ergotoxine respectively. The action of kurchicine closely resembles conessine in this respect.

Amphibian and mammalian heart—Intact frog's heart when perfused with a solution of 1 in 50,000 kurchicine shows little or no change, but with higher concentrations a definite depressant effect is obtained.

In myocardiograph experiments in cats intravenous injections of the alkaloid in doses of 1 mg to 2 mg produce a slight stimulation of the auricles with dilatation of the ventricles. With larger doses, such as 4 mg, this effect is much more pronounced.

Isolated heart of rabbits and kittens were perfused with warm oxygenated Locke's solutions (pH 7.4). The alkaloid in dilutions of 1 in 1,000,000 has no effect whatever on the heart but with 1 in 100,000 the ventricular beats are slowed and finally a definite heart block is produced. The amplitude of the ventricular beats is not affected at first but a little later there is distinct depression in the force of the beats. With stronger concentrations, i.e., from 1 in 20,000 to 1 in 5,000, the decrease in the force and frequency of the beats is very marked and complete heart block is quickly produced, the auricles and the ventricles beating independently.

Comparative effects of kurchicine and emetine on the isolated heart were also studied. Kurchicine hydrochloride in concentration 1 in 50,000 has no depressant effect upon this organ while emetine 1 in 100,000 to 1 in 200,000 dilutions produces a powerful and marked effect.

After administration of 2 mg of kurchicine hydrochloride the heart as recorded by the cardiometer shows a well-marked depression due to dilatation of the organ as a result of which the heart gradually loses its tone but the volume does not return to normal for a long time.

Action upon the blood vessels—It has been observed that the alkaloid, even in small doses, produces a marked and sustained fall in blood-pressure, both before and after the sympathetic nerve-ending.

paralysed with eigotoxine and with nicotine respectively. Similar effects were also obtained after section of both the vagi and after paralysing the nerve-endings with atropine. All these experiments tend to show that the fall is due to the direct action of the alkaloid on the blood vessels. Perfusion of the blood vessels of the frog using the Trendelenburg's technique with 1 in 100,000 solution at pH 7.4 produces an increase in the flow of the perfusate showing that the blood vessels are dilated. Perfusion of the splanchnic vessels of a cat with a solution of 1 in 25,000 to 1 in 100,000 at pH 7.4 also shows a well-marked dilatation of these vessels.

Volume of organs—An injection of 3 mg of the alkaloid produces an increase in the volume of the intestine and of the kidney, whereas the volume of the limbs and of the spleen shows a decrease after a small initial increase. This probably corresponds to the fall of the blood-pressure.

Uterus—The virgin uterus of a cat *in situ* always shows a definite tonic contraction of this organ when 3 mg to 5 mg of kurchicine hydrochloride are given intravenously.

The isolated uterus (virgin) of the rabbit is stimulated with dilutions as high as 1 in 500,000 and tonic contraction is produced with lower concentrations such as 1 in 200,000 to 1 in 100,000. The general tone and amplitude of the rhythmic movements is always increased.

SUMMARY AND CONCLUSIONS

From the experimental data obtained it would appear that the alkaloid kurchicine is a general protoplasmic poison like emetine. The M L D in white mice is 38.12 mg, in guinea-pigs 64.3 mg, per kilo body-weight. Intravenous injections of the hydrochloride of the alkaloid in animals produce a well-marked fall in blood-pressure. The heart is depressed but this depression cannot totally account for the fall in blood-pressure. With toxic doses there is definite slowing of the heart followed by complete heart block probably due to the direct depressant action of the alkaloid on the auriculo-ventricular bundle of His. The alkaloid produces a marked dilatation of the vessels of the splanchnic area.

The respiration is at first slightly stimulated probably secondary to the fall in blood-pressure, but with large doses it quickly stops, the heart going on beating for a long time after.

The plain muscles of intestine and uterus are stimulated even in as high a dilution as 1 in 500,000.

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ON *BACILLUS PESTIS* NEW TECHNIQUES IN SEROLOGY

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THE serological methods employed in the study of *Bacillus pestis* have so far been very unsatisfactory

The organism, as grown ordinarily at room temperature, is auto-agglutinable in normal saline and thus useless for an agglutination test. Attempts at obtaining a reliable and constant suspension by incubation at blood-heat (after Dieudonne and Otto, 1927), by suspending the growth in distilled water (after Kolmer, 1924), by sand filtration (after d'Aunoy, 1923), and by formalinizing the suspension (after Batchelder, 1929) did not yield useful and repeatable results in our hands. The first three methods incidentally make use of the living organism and are potentially dangerous.

In studying the effect of age on the organism it was found that sediments from old broth cultures re-suspended in normal saline always yielded stable suspensions. Such suspensions, however, were found to agglutinate with normal sera in low dilutions. This defect was found to disappear on the addition of very small quantities of formalin.

Complement-fixation does not appear to have been employed in the study of *Bacillus pestis* at all. The reaction, however, was found to give clear-cut and graded results.

In this communication the techniques of agglutination and complement-fixation will be described in detail and their applications indicated. Preliminary

observations on the agglutinating suspension, the complement-fixing antigen and the corresponding anti-bodies will also be made

I THE AGGLUTINATION A DEBRIS AGGLUTINATION

1 The reagents

(i) *The debris suspension*—The deposit from Haffkine's plague vaccine, especially after eight weeks, is re-suspended in a large test-tube capable of holding about 100 ml of fluid without wetting the plug, by means of beads, in normal saline solution (0.9 per cent and phenol (Merck) 0.25 per cent have been found to be best). The final suspension is adjusted to lie between those of No. 2 and No. 3. The suspension left at room temperature overnight will be found to be standing with a clear supernatant and a small but compact deposit on the bottom. The movement involved in taking the tube out of the rack and the top homogeneous again without disturbing the deposit is pipetted off without loss. Its opacity is that of the vaccine yields about

vaccine has recently been fully described usually a heat-killed (at 55°C for 15 minutes) freshly isolated from the heart of a mouse to plague, in acid-digest (about 27°C in Bombay except acid-digest broth is essentially the same) diluted and neutralized to contain 0.5 per cent, have a pH of 6.8. For the purpose of growing for eight weeks before it is killed was the customary period of growth of a

has been described by Naidu, Jung and Kamakaka as a view to its therapeutic application in cases of plague. The serum obtained from sheep, buffaloes and bullocks after 24 hours with agar cultures of *B. pestis* suspended in the a six weeks old culture of the same organism in acid-digest are grown at room temperature

2 The method of charging the tubes

thinning a thin debris suspension still further, the serum dilutions are ten times as strong as the final dilution aimed at. One volume of such a dilution is added to nine volumes of the suspension. Small tubes with sloping sides, like those used in Dreyer's rack, show the resulting flocculation much better than do the big tubes like those used in a Wassermann test. The big tubes, however, are useful in detecting an early granularity of the suspension.

3 The ensemble

Tube number	1	2	3	4	5	6	7
Serum dilution, 1 vol of —	1 in 50	1 in 100	1 in 200	1 in 300	1 in 400	1 in 500	0 (saline only)
Suspension	9 volumes per tube						
Final dilution of the serum	1 in 500	1 in 1 000	1 in 2,000	1 in 3,000	1 in 4,000	1 in 5,000	No serum (suspension control)

The volumes are measured with a 1 c c pipette worked with a rubber-teat. For a small tube such as is used in a Dreyer's rack 0.1 c c of the serum dilution and 0.9 c c of the suspension suffice, while for a big tube such as is used in a Wassermann test 0.2 c c of the serum dilution and 1.8 c c of the suspension are used.

The tubes are shaken individually, bubbles are expelled from the small tubes, and the rack is left in the incubator. The reaction is read in three hours, six hours and next day. Generally a well-marked reaction occurs in three hours.

4 Reading of results

The appearance is unmistakable. The readings are —

++ = Masses of flocculation in a non-opalescent or almost non-opalescent fluid

+ = A well-marked flocculation in a still definitely opalescent fluid

± = A definite flocculation in some part without a definite alteration in the opalescence of the fluid elsewhere

— = No change when compared with the original suspension

(?) denote a doubt with regard to a sign. A + (?) is not quite a + though definitely more advanced than a ±. Used in comparisons.

One of the immunized sheep in the Haffkine Institute (sheep No. 33) has for the last two years yielded a serum giving a ++ reaction in from 1 in 1,000 to 1 in 2,000, a + reaction up to 1 in 4,000 and a ± reaction up to 1 in 6,000.

5 The specificity of the reaction

Normal sera from sheep, bullocks and buffaloes have given a negative reaction. Anti-rabic sera from sheep and buffaloes, the only other anti-sera available from

these animals so far, have also given a negative reaction. Debris suspensions made from some other members of the *Pasteurella* group, namely, *B. avisepticus*, *B. bovisepiticus*, a bacillus of deer septicæmia and *B. pseudotuberculosis rodentium* have given poor reaction with the anti-serum, with the exception of the debris from the last organism. The reaction in this case is as strong as with a *B. pestis* debris so far as a ++ reaction is concerned. Generally, later it fails in a + and a ± reaction. A fresh suspension in saline of *B. pestis* removes the anti-body from the serum and completely abolishes the reaction. Other members of the *Pasteurella* group fail to do so. This item is dealt with separately under the next heading.

6 The absorption test

Cultures of *B. pestis*, *B. avisepticus*, *B. bovisepiticus*, Bacillus of deer septicæmia, *B. pseudotuberculosis rodentium* and Bacillus of bovine lymphangitis (Kishnamurti's organism) are grown on blood-agar, in the incubator, for 24 to 48 hours, washed off with normal saline and killed by heat at 60°C for half an hour. The opacity of the suspensions so made is adjusted to lie between those of No. 9 and No. 10 opacity tubes.

In small sterile tubes, suitably labelled, one volume of a 1 in 10 anti-plague serum, giving a ++ agglutination in 1 in 1,000 dilution, is added to nine volumes of the suspension from each organism. 0.1 c.c. of the serum added to 0.9 c.c. of the suspension will suffice. The mixture is left in the incubator overnight. A serum control made with saline only is also incubated for the same period.

When the agglutinating titre, for a ++ reaction, of a serum is 1 in 2,000, one volume of a 1 in 20 dilution is added to the suspension instead of a 1 in 10 dilution.

Next morning the tubes are centrifuged until the supernatant fluid is clear. One volume of this clear fluid from each tube is added to nine volumes of the debris suspension in suitably labelled agglutination tubes. A debris suspension control, made with the debris suspension and saline only (9:1), is also prepared. Precautions of sterility are not needed at this stage. The tubes are incubated. Results are read in three hours, six hours and next day. Generally, it is possible to record the results in three hours.

The tubes containing the serum absorbed by *B. pestis* is found to be as free from agglutination as the debris suspension control, the fresh suspension of *B. pestis* having absorbed the homologous anti-body. The tubes containing the sera absorbed by the other organisms show a high degree of agglutination. That there should be differences between themselves and between them and the serum control is to be expected from a group relationship. So far we have not studied these differences quantitatively.

We have employed this test in identifying fresh cultures of *B. pestis* from rats sent for examination. A definite decision can be arrived at in 48 to 72 hours. The fact assumes significance when the importance of movements of troops and ships from a port like Bombay and the slowness of the animal test in confirming a suspicious looking culture are considered.

Heating at 60°C for half an hour is employed merely to kill the organisms and make them safe for handling. It does not promote the absorption of the anti-serum.

We have found that the amount of the serum absorbed by a suspension of heated and killed bacilli is the same as that absorbed by a suspension of unheated and living bacilli. This finding is opposed to that of Schutze (1932) who while admitting that agglutination of *B. pestis* 'has always been accompanied by difficulties' claims to have found evidence, by agglutination tests, for two types of anti-sera against *B. pestis*, (i) an 'anti-somatic' type reacting with the naked bodies of the bacilli (grown at 27°C) only and (ii) an 'anti-envelop' type reacting with 'enveloped' bacilli (grown at 37°C) only. The 'anti-somatic' serum, according to him, can only act on the enveloped bacilli when the envelope has been damaged by heating and steaming. The serum used by us is obtained by injecting animals with cultures grown at room temperature only (27°C) and therefore must be mainly an 'anti-somatic' serum. The bacilli grown at 37°C are thicker than those grown at 27°C, show the characteristic gelatinousness while forming a suspension in saline, are not salt-sensitive and must be 'enveloped'.

Further, the bacilli grown at 37°C absorb the anti-serum as well as those grown at room temperature (27°C). This fact is demonstrated by reducing the densities of the suspension and ascertaining the minimal limit needed for absorption.

The question of the unity of the anti-serum, as opposed to a duality described by Schutze, is significant where the preparation of a prophylactic vaccine against plague and of a therapeutic serum for acute cases of the disease are concerned.

7 Applications of the techniques of agglutination and absorption

Besides the identification of an unknown culture suspected to be *B. pestis* and the demonstration of the unity of the anti-body produced as a response against the organism, as outlined under the previous heading, the techniques could be applied to (i) a differentiation of strains of *B. pestis*, if any, (ii) a standardization of the plague vaccine, (iii) a measurement of the potency of a therapeutic serum, if a correlation with protection experiment could be established, and (iv) a determination of group relationship between the members of the Pasteurella group.

II THE COMPLEMENT-FIXATION

1 The reagents

(i) The hæmolytic system as prepared for a Wassermann reaction and used in the same proportions. For the titration of the complement method No 4 of the Medical Research Council (1928) has been followed.

(ii) Saline as used in a Wassermann reaction.

(iii) *The antigen* —An ampoule of Hafkine's plague vaccine, especially grown for eight weeks, as described under Agglutination, is centrifugalized until the supernatant fluid is clear. The fluid is pipetted off and filtered through an L₃ candle. The filtrate is diluted with an equal volume of saline.

(iv) *The serum* —As described under Agglutination, diluted 1 in 10. Inactivation at 55°C for 10 minutes is only necessary if the serum is markedly anti-complementary. Only sheep serum can be inactivated without destroying the anti-body.

2 *The ensemble*

Reagents	Serum control tubes —		Antigen control tubes —			Test proper tubes —		
	1st	2nd	1st	2nd	3rd	1st	2nd	3rd
Complement, M H D in 1 vol	2	3	5	6	7	10	12	14
Serum dilution	1 vol		Nil			1 vol		
Antigen	Nil		1 vol			1 vol		
Saline	1 vol		1 vol			Nil		

(a) Left at room temperature for one hour (b) Shaken thoroughly to re-suspend a precipitate resulting from the action of the precipitin in the serum (c) Left in water-bath at 37°C for 45 minutes (d) Left at room temperature again for 15 minutes (e) Shaken again to resuspend the precipitate (f) Sensitized red cell suspension, 1 vol, added to all the tubes (g) Left in water-bath for half an hour (h) Lysis and inhibition of lysis noted (i) Left in the cold overnight (j) Traces of lysis noted

For weak sera smaller doses of the complement are used in the test proper, e.g., 8, 10 and 12 M H D or 7, 8 and 10 M H D instead of 10, 12 and 14 M H D. To detect the merest trace of the immune body in a very weak serum the dilution of the serum is lowered, e.g., a 1 in 7 or 1 in 5 dilution is used instead of a 1 in 10 dilution.

For strong sera the dilution of the serum can be raised from 1 in 10 to 1 in 12, 1 in 15 or even higher. The dose of the complement need not be raised above 14.

A large number of repetitions has shown that constant and comparable results can be obtained by keeping to the above figures.

3 *Reading of results*

M H D of the complement in the fully lysed serum control *plus* the M H D in the fully lysed antigen control *minus* 1 equals the M H D needed for a complete lysis when the serum and the antigen are put together (both being anti-complementary). Any amount needed for an incipient lysis above this figure *plus* 1 represents the complement-fixation measured in M H D.

The reason for subtracting 1 is this. Had the two controls (the antigen control and the serum control) been mixed together as they stood in the tubes there would have been two volumes of the red cell suspension to lyse while in the test proper there is only one volume of the red cell suspension to lyse.

The reason for adding 1 is this. The amount of complement needed for a complete lysis would, of course, produce a complete lysis in the tube containing the

amount, when there is, with this amount, an inhibition of lysis instead or only an incipient lysis which amounts practically to an inhibition, 1 M H D has already been fixed, thus 1 M H D must be added to the total above the figure needed for the incipient lysis. The following examples are typical —

Sera	Serum control tubes —		Antigen control tubes —			Test proper tubes —		
	1st (2 doses)	2nd (3 doses)	1st (5 d's)	2nd (6 d's)	3rd (7 d's)	1st (10 d's)	2nd (12 d's)	3rd (14 d's)
A	—	—	—	—	—	+	+	+
B	—	—	=	—	—	+	+	+(?)
C	—	—	±	±	—	+	+	±
D	—	—	—	—	—	±	±	±
E	—	—	—	—	—	—	—	—
F	±	—	±	—	—	+	+	+
G	+	+	+	—	—	+	+	+

Calculations

Serum A

M H D fixed = $14 - (2 + 5 - 1) + 1 = 9$ at least (the end point not reached)

Serum B

M H D fixed = $14 - (2 + 6 - 1) + 1 = 8$ ‘?’ indicates a trace of lysis (an incipient lysis) and a reaching of the end point

Serum C

M H D fixed = $12 - (2 + 7 - 1) + 1 = 5$

Serum D

‘Irregular’ reaction observed

Serum E

Negative

Serum F

M H D fixed = $14 - (6 + 3 - 1) + 1 = 7$ at least

Serum G

Results cannot be read Should be repeated with higher doses in the controls

A remark may here be made on the 'irregular' reactions They occur contrary to arithmetical considerations In serum D a \pm reaction is obtained with 10 M H D of the complement This lack of complete lysis indicates a lack of only a fraction of an M H D With 12 M H D there should not be any inhibition of the lysis at all Hence the 'irregularity' Such reactions, apparently unrecognized by other workers in complement-fixation, are quite frequent occurrences One of us has drawn attention to them previously in this *Journal* (Greval, 1930) For quantitative record we take cognizance of regular reactions only

4 *Specificity of the reaction*

Combinations of (a) normal sera and a filtrate as described above and of (b) immune sera and a filtrate prepared from carbolyzed acid-digest broth have given negative results with the lowest doses of the complement Anti-rabic sera from sheep and buffaloes, the only other anti-sera from these animals available so far, have also given negative results Filtrates prepared from some other members of the *Pasteurella* group, namely, *B. avisepticus*, *B. bovissepticus*, *Bacillus* of deer septicæmia and *B. pseudotuberculosis rodentium* have given a poor reaction with the anti-serum with the exception of a filtrate from the last organism The reaction in this case has been well marked but always less so than with a filtrate of the same age from *B. pestis* Suspensions of *B. pestis* absorb the anti-body from the serum and abolish the reaction On absorption more will be said under separate headings

5 *Post-absorption complement-fixation*

When the plague anti-serum is absorbed with *B. pestis* and other members of the *Pasteurella* group, as detailed under the absorption test, it is found to have lost its power of fixing complement completely after absorption with *B. pestis* and incompletely after absorption with the other members The incomplete loss is both qualitative and quantitative, the reaction becoming irregular before disappearing

6 *Post-fixation agglutination*

The supernatant colourless fluid in a positive tube, after complement-fixation, represents a 1 in 40 dilution of the original anti-serum When this colourless fluid is pipetted off, diluted further and tested for its agglutinating titre, it is found to have suffered little loss of the titre when compared to a similar dilution of the original anti-serum kept as a control and subjected to the same conditions of incubation Evidently, the reaction involved in agglutination (and absorption) affects both the agglutinating and the complement-fixing anti-bodies while the reactions involved in complement-fixation affects only the complement-fixing anti-body

7 *Precipitation occurring during complement-fixation*

As observed in the Ensemble a well-marked precipitate occurs during the process of complement-fixation. The strength of the serum in the tubes at the time of the observation (before adding the red cell suspension) is 1 in 30. The reaction is quite independent of the complement present. The deposit unless shaken up at the stages already indicated is likely to be confused with a deposit of the red cells such as occurs in an incomplete hæmolysis.

8 *Application of the technique of complement-fixation*

Inasmuch as there are indications that the complement-fixing anti-body is different from the agglutinating anti-body, its correlation with protection experiments would be informative. The technique could also be used to confirm the results of agglutination and absorption generally. As a mere means of measuring the titre of the anti-serum, without reference to a definite protection afforded by such serum, the technique is inferior to that of agglutination.

III PRELIMINARY OBSERVATIONS ON THE REAGENTS

1 *The debris suspension used in agglutination*

The deposit in a broth culture, killed by heat and carbolyzed, begins to show the properties of forming a stable suspension, reacting with the anti-serum, after three weeks of growth at room temperature. Deposits from eight weeks old cultures besides yielding more of the suspension react more quickly with the anti-serum, the reaction being complete in six hours at 37°C. A four weeks old culture is, however, more easily available from the standard Haffkine plague vaccine and can be used if the results are to be read after twelve to eighteen hours. In the early part of this work deposits from standard vaccines only were employed. In leaving the test standing for longer than six hours a certain amount of compact and yellowish sedimentation, occurring in all the tubes including the control, must be excluded. Its appearance is distinctive.

Microscopically the material forming the opalescent suspension consists of debris. When a flocculus is spread on a slide it films badly like mucus.

The addition of formol is essential. Although the quantity used (0.05 per cent) appears to be negligible it can be demonstrated by colour reactions in a suspension kept for months in a flask plugged with cotton-wool only. Phenol is added as an antiseptic. By its use the suspension can be handled more easily and kept longer. It also abolishes the risk of growth in the test kept in open tubes overnight. We keep saline containing the requisite quantities of formol and phenol ready in flasks. Our suspension is also made in bulk in a strong Pyrex flask containing beads. A night before use the flask is shaken and the suspension transferred to a large tube which is kept upright for the night.

Not all deposits from broth cultures of *B. pestis*, three weeks or over old, will form suitable suspension. The majority of the cultures, however, will do so. When one sample from a large stock is found to be satisfactory, the whole of the stock is satisfactory. The age of the bottled vaccine does not seem to affect the agglutinating

properties of the suspension. Similar suspensions have been made from other members of the *Pasteurella* group. Their growth at room temperature is less profuse than that of *B. pestis*.

2 *The absorbing suspension used in the absorption test*

The density of the fresh suspension of the bacilli washed off an agar surface with saline, has been found to be optimal when its opacity lies between those of No 9 and No 10 Brown's tubes. Working with a 1 in 1,000 dilution of an anti-serum, producing a ++ agglutination in a 1 in 1,000 dilution a distinct change in reaction occurs when the density of *B. pestis* suspension is halved. The suspension is not now rich enough to abolish the agglutination after the absorption. On the other hand, in the case of the other organisms of the *Pasteurella* group, studied so far, even a doubling of the density of their suspensions has not enabled them to absorb the anti-plague serum completely like *B. pestis*.

The density may be varied, in fact must be varied, for quantitative estimations. Certain 'strains' of *B. pestis*, for instance, may absorb more anti-serum than others.

Possibly the time taken in absorption may also prove to be of value in the differentiation of 'strains'.

For a comparison of the opacity with Nos 9 and 10 tubes a thick line with well-defined edges serves the purpose better than the print. The tube containing the suspension under trial is laid between the two tubes on the line and the sharpness of the edges of the line compared.

3 *The anti-serum*

Most of the work reported here was done with sheep serum. Buffalo or bullock sera have not been found to be so potent serologically as the sheep serum. Besides the complement-fixing anti-body of the sheep serum is thermostabile while that of the buffalo or bullock sera is thermolabile, disappearing completely when kept at 55°C for 10 minutes. This was also found by one of us (Gieval, 1933), to be the case with anti-rabic sera from sheep and buffaloes.

The sera which though sterile form heavy deposits at the bottoms of the tubes or of the ampoules, when kept in a cold chamber, are found to have suffered a loss of the anti-bodies.

In sera which have deteriorated from age, effect of heat, lack of sterility or some other cause, the complement-fixing anti-body appears to be damaged more than the agglutinating anti-body.

4 *The filtrate used in complement-fixation*

The supernatant fluid from a vaccine ampoule is filtered to get rid of the debris of the bacilli which, besides being anti-complementary, interferes with the fixation of the complement by the anti-serum, by absorbing the latter.

Complement-fixing anti-body appears in the filtrate from a culture at the end of the first week. The anti-complementary titre of the filtrate is low but the

reaction of fixation is irregular and weak. Every added week to the age of the culture strengthens the reaction of fixation but unfortunately increases the anti-complementary titre of the filtrate at the same time.

The anti-complementary titre of the filtrate shows an extreme irregularity in inhibition of lysis. For instance, in a series of four tubes charged with eight weeks filtrate and 11, 12, 13 and 14 M H D of complement the lysis, well marked in the first tube, is still incomplete in the fourth tube in spite of the fact that between the first and the fourth tubes there is a difference of 3 M H D of complement. This irregularity is the chief difficulty in obtaining a good reading of the fixation. It has been found that diluting the filtrate with an equal volume of saline reduces the irregularity considerably. Trials with various filtrates and a constant serum have shown that maximum readings are obtained with a eight weeks filtrate diluted with an equal volume of saline.

The quantity of the complement-fixing antigen in the diluted filtrate is about the minimum needed for a regular reaction with a 1 in 10 dilution of a potent anti-serum. This means that if such a serum, giving a regular reaction, gives a certain reading of fixation in a 1 in 10 dilution, it is not expected to give a higher reading in a 1 in 5 dilution.

As observed under complement-fixation, the filtrate also yields a distinct precipitate with the anti-serum starting as an immediate cloudiness which thickens with incubation. We have not studied the reaction further.

The bottled vaccine from which the filtrate is obtained appears to keep unchanged for this purpose for at least four months. Probably it keeps for a much longer period. Temperature and light do not affect it. In two samples of one year old vaccine, one kept at room temperature and the other in a refrigerator, the quantity of the antigen was exactly the same, as shown by a complement-fixation with a constant serum.

A filtrate from a four weeks old culture is readily available from the standard Haffkine's plague vaccine and can be used undiluted in determining the reaction. Because of a high anti-complementary titre of an undiluted filtrate the readings obtained by its use are low.

SUMMARY

1. A technique of an agglutination of the debris of *B. pestis* with plague anti-serum has been described. Besides measuring the titre of the serum it makes the identification of *B. pestis* possible with the aid of an absorption test.

2. A technique of a complement-fixation with plague anti-serum has been described. The antigen is a filtrate from broth cultures of *B. pestis*. A relationship between the agglutinating anti-body and the complement-fixing anti-body has been pointed out.

3. Preliminary remarks on the reagents and the reacting bodies have been made.

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difference in the action of all these three drugs (1) on the course of infection, (2) on the changes of the parasite, and (3) the pathological alterations in some of the chief organs and tissues of the infected animal. It may be stated once for all that these three compounds when administered during the course of an acute infection early enough are capable, each in its own way, of preventing the death of the animal. All the three drugs, however, fail in completely ridding the animal of the infecting parasite, in other words, even after a sufficiently long course of the drug administered in adequate doses, the relapses come on periodically, with the return of the sexual and asexual forms of the parasite and these in their usual virulence.

Before considering the mechanism involved in each case, it may be briefly stated that in an acute infection the rings increase day by day and each crop of the invading parasites in the ring form goes through the normal cycle of the asexual development of the parasite,—from the trophozoite to the schizont with a large number of merozoites. This rapid flooding of the blood by the parasites leads to an alarming destruction of the red blood corpuscles, resulting in an intense anæmia and hydræmia until no more blood corpuscles are available for the attack, and the animal dies with the load of fully developed merozoites then present. These blood changes are best seen in a microphotograph (Plate XVI, figs 1 and 2).

Quinine—When the invading parasites are still young and not beyond the early trophozoite stage, provided the proportion of the invaded corpuscles is well below 40 per cent, if quinine in $2\frac{1}{2}$ grain doses is administered the rapid growth of the trophozoites and also the undue formation of the merozoites is checked and a sort of balance is established for the new formed red blood corpuscles to keep pace with the subdued multiplication of the parasite. The trophozoite and the host cell, along with the merozoites, shrink and appear to be destroyed *in situ* and phagocytosed. Some of the few rings which escape this fate slowly develop into trophozoites, and gametocytes now begin to make their appearance. This quiescent state continues for a few days when again there is a recrudescence of rings periodically with a tendency towards an acute attack and its attendant dangers, unless this is also prevented by similar administration of quinine as before, after dealing thus with the first two or three relapses, a chronic state is brought about, when the production of the numbers of merozoites is sufficiently reduced and gametocytogony well established (Plate XVIII, figs 7, 8, 9 and 10).

Atebrin—In the case of atebrin, the rings seem to be attacked directly, even in such a small dose as 0.025 g. as they are inhibited in their progress into even the trophozoite stage and therefore the destruction of the rings is more rapid than under quinine, in which case 24 hours after the exhibition of the drug the rings are not prevented from growing into trophozoites though in a stunted form. The blood after two days of atebrin is practically cleared of the rings, but they reappear after about ten days of the last day of the exhibition of atebrin, even when the drug is continued for 15 consecutive days, and the same good result is obtained by another course of atebrin and the animal is saved each time. In these earlier relapses the rings accumulate slowly and take two or three days before the danger point is reached. When, however, this treatment is carried on for some time for two or three weeks the subsequent recrudescence is characterized by the return of a large number of rings about ten days after the last day of atebrin, and then there is the danger of losing the animal unless immediate steps are taken to control this

PLATE XVI

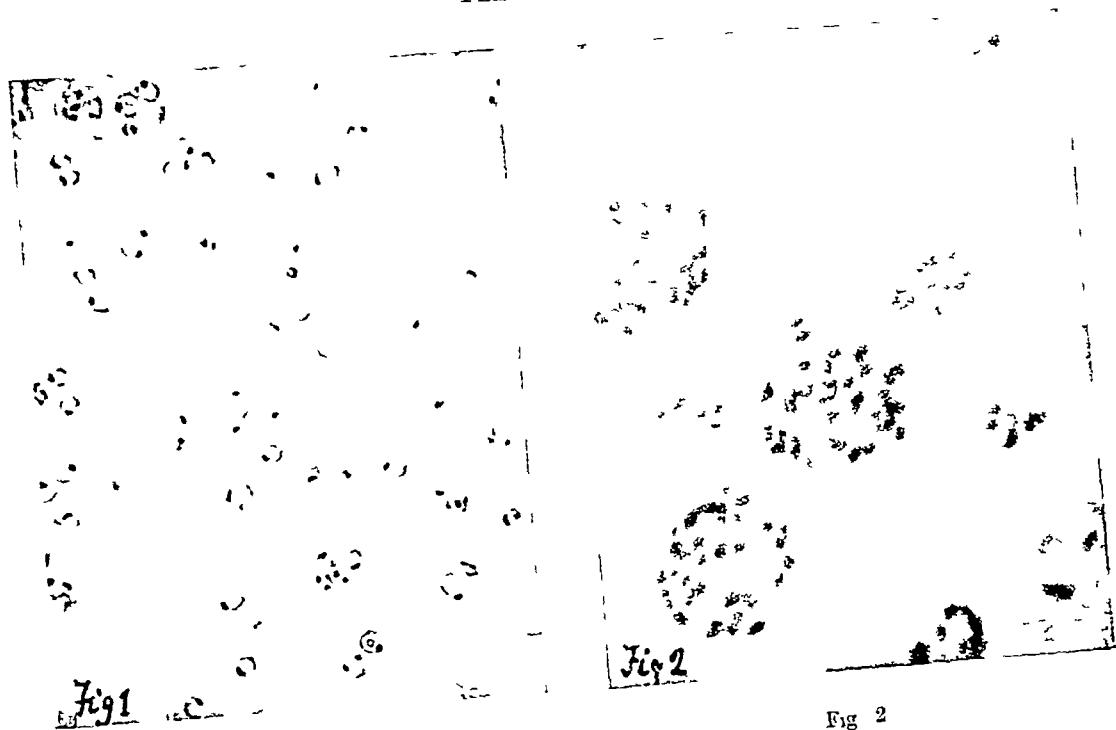


Fig 1

Fig 2

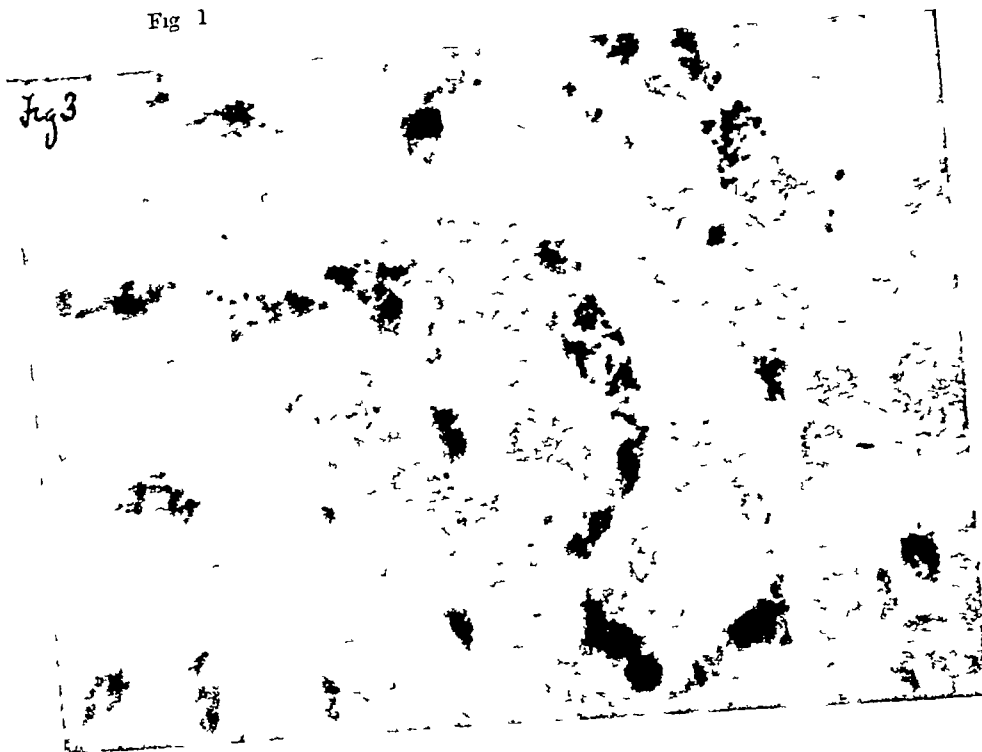


Fig 3

- Fig 1 Peripheral blood of *Macacus rhesus*, fifth day after infection
 " 2 Peripheral blood of the same, sixth day after infection, just before death
 " 3 Section of liver P M of *Macacus rhesus* in malarial infection

PLATE XVII

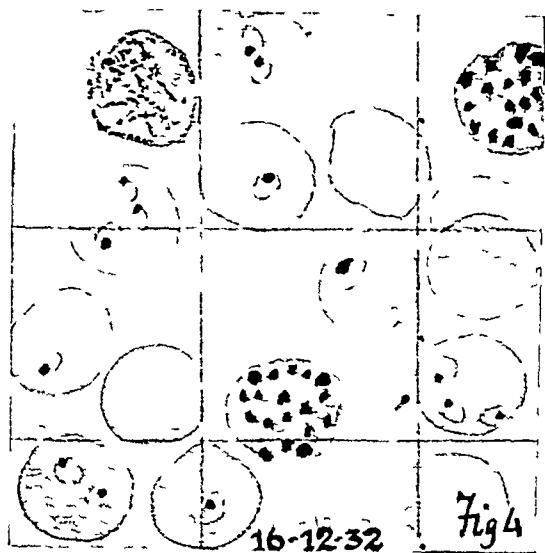


Fig. 4

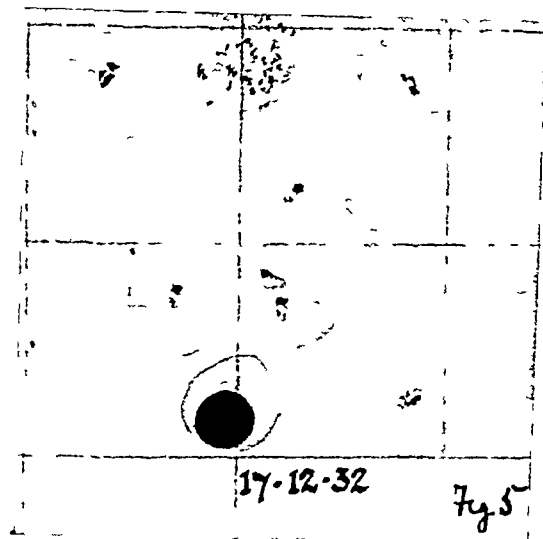


Fig 5

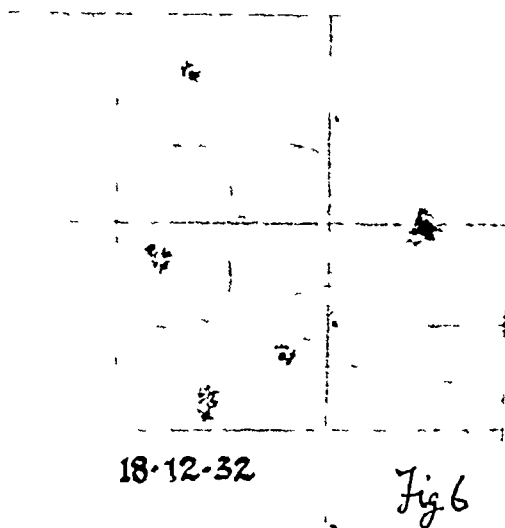


Fig 6

Action of atebryn in the *Macacus rhesus* infected with malaria

- Fig 4 Peripheral blood, fifth day after infection just before administration of atebryn
 „ 5 Peripheral blood of the same, sixth day after infection Twenty four hours after the administration of one dose of atebryn (0.05 g)
 „ 6 Peripheral blood, seventh day after infection Forty eight hours after (had two doses by now)

PLATE XVIII

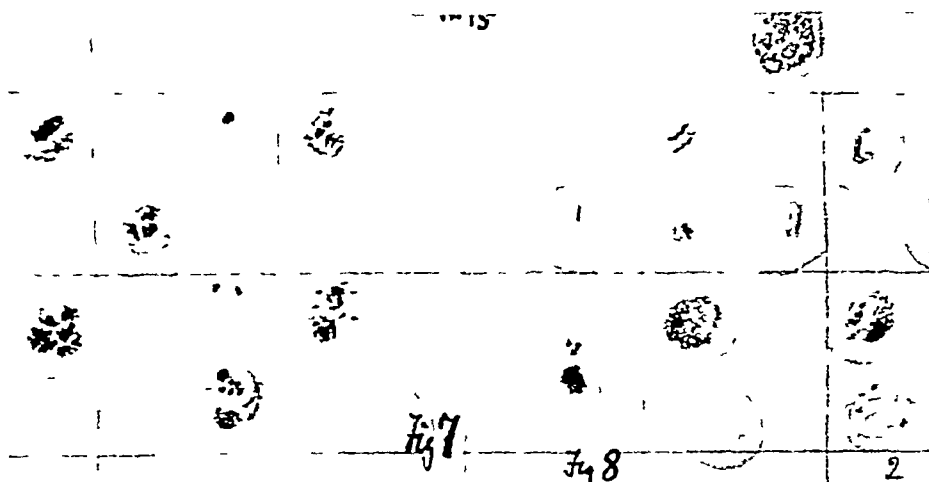


Fig 7

Fig 8

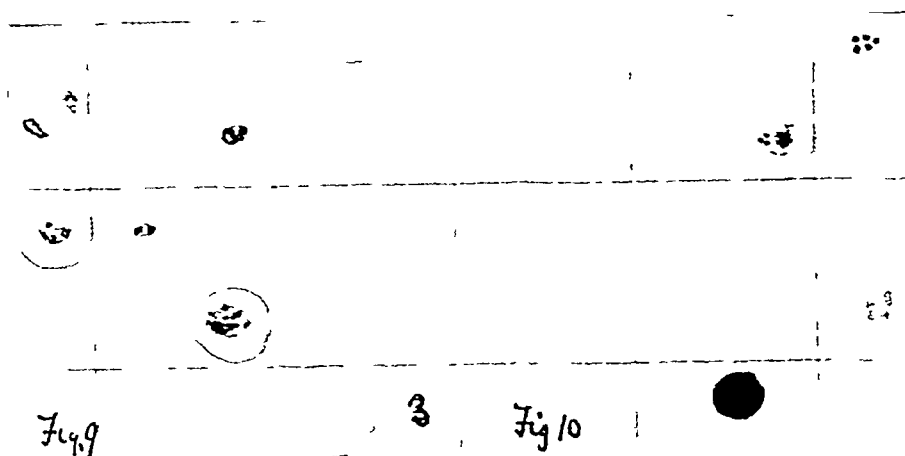


Fig 9

Fig 10

Action of quinine in *Macacus rhesus* infected with malaria

- | | | |
|-----|----|--|
| Fig | 7 | Peripheral blood, fifth day after infection |
| " | 8 | Peripheral blood, 24 hours after the first dose of quinine (2.5 grams per os) |
| " | 9 | Peripheral blood seventh day after infection Forty-eight hours after the first dose of quinine |
| " | 10 | Peripheral blood, eighth day of infection Seventy two hours after the first dose of quinine (had by now three doses) |

PLATE XIX

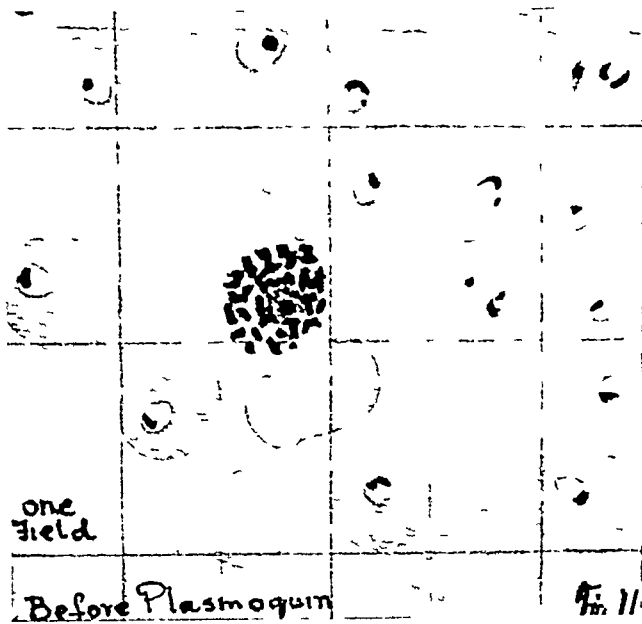


Fig 11

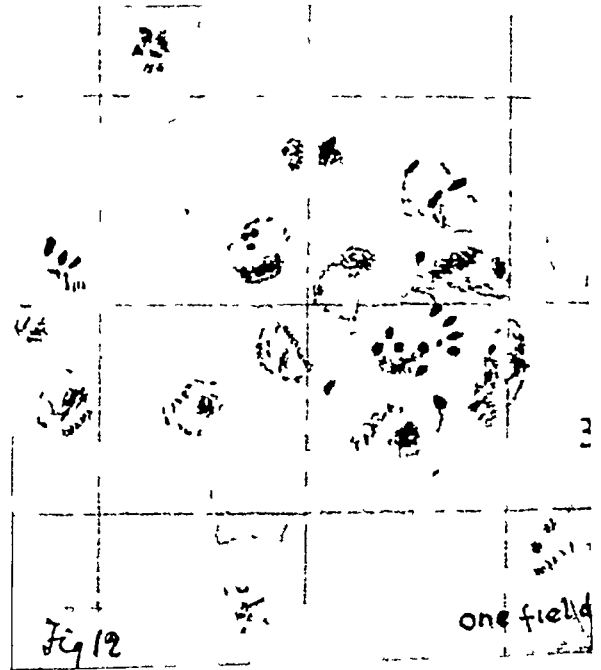


Fig 12

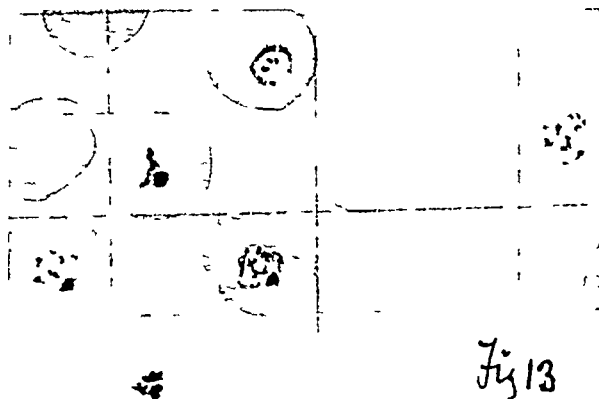


Fig 13

Action of plasmoquin in *Macacus rhesus* infected with malaria

- Fig 11 Peripheral blood, fifth day after infection, before the administration of plasmoquin
 " 12 Peripheral blood, sixth day after infection, 24 hours after the first dose of plasmoquin (0.005 g per os).
 " 13 Peripheral blood, seventh day after the infection (had two doses by now)

sudden invasion by repeating the atebirin administration (Plate XVII, figs 4, 5 and 6)

Plasmoquin —The action of plasmoquin on the asexual cycle is of interest as it resembles that of quinine in its slowness, but is more potent even in small doses. Thus, in spite of the enormous number of parasites in even an advanced trophozoite stage, and in spite of the dangerous sign of cyanosis, the parasites are destroyed and the animal is saved with even a few days treatment with this compound. The secondary anæmia, however, is more intense than under atebirin or quinine, but the rings return early, so that after a four days interval of a long course of plasmoquin (lasting seven consecutive days and four days after a gap of three days) the blood shows four or five rings in each field and the animal runs the risk of succumbing to the effect of a relapse, unless measures are adopted to control the parasite immediately by any of these three drugs. Some of these facts are best illustrated in Plate XIX, figs 11, 12 and 13.

These observations lead one to the conclusion that no matter which of these compounds is used there is always a return of the parasites with this difference that the state of equilibrium leading to a chronic infection is brought about more effectively with fewer courses of quinine than with the other two products.

Pathology

The pathology of the disease in the acute attack in treated and untreated cases alike is chiefly directed to blood changes. As the parasite progresses in its cycle *in vivo* in an acute infection and then recurrently floods the blood in repeated crops, the red blood cells are attacked and destroyed by millions per cubic millimetre every day in spite of their being replenished partially in the early stage and an intense hydræmia and oligocythæmia are induced by the time the danger signs establish themselves, the blood is dark-greyish in colour and watery. The morphological changes in the red blood corpuscles free from parasites, to state briefly, are vacuolation and pallor with a fair sprinkling of poikilocytes, polychromatophiles, and normoblasts. White blood cells are increased with excess of large mononuclears, many of which contain red blood cells, phagocytosed parasites and striking masses of dark pigment.

The spleen is large and soft and almost black on section. The organ is crowded with infected red blood cells with the parasites in all stages of development, and is packed with confused masses containing parasites, red blood cell debris and abundant pigment, a state indicative of acute red infarction, leaving only small islets of normal spleen-tissue.

The liver is also enlarged and congested with pale blood and in section shows the usual changes found in infection, e.g., cloudy swelling and fatty changes. The blood vessels are full of red blood cells many of which contain parasites but the most remarkable change is the distribution of pigment which occupies the lymph spaces between the hepatic cell sinusoids and is all concentrated in the Kupffer cells *vide* Plate XVI, fig. 3.

The bone-marrow is red and full of regenerating red blood corpuscles, some of the fully formed red blood cells being full of parasites. The brain and kidney are also loaded with parasites, in their capillaries, and in severe infections one finds hæmaturia rather than hæmoglobinuria and this is the result of glomerular vessels

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being plugged with masses of red blood corpuscles containing the parasites and the subsequent acute thrombotic nephritis

The pathological changes in the chronic disease have not yet been worked out completely, as all the animals so far treated are fairly well except for the secondary anæmia induced by the earlier attacks

The authors are indebted to Lieut-Colonel S S Sokhey, I M S, Director, Hafikine Institute, for kindly placing all laboratory facilities at their disposal

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THE TRANSMISSION OF KALA-AZAR TO HAMSTERS BY THE BITE OF THE SANDFLY *PHLEBOTOMUS* *ARGENTIPES*

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THIS is a report of a series of experiments during which the causative organism of kala-azar, *Leishmania donovani*, was successfully transmitted from human patients suffering from kala-azar to Chinese hamsters, *Cricetulus griseus*, by the agency of the sandfly, *Phlebotomus argentipes*, via the insect's proboscis

Hitherto all attempts to transmit this infection to human volunteers and other animals by means of the bite of this fly have failed, although the workers on kala-azar at the Calcutta School of Tropical Medicine and in Assam have directed their main energies on work with this particular insect, since the discovery of the development of the parasite in its gut (Knowles, Napier and Smith, 1924) In the case of the hamster one successful transmission has been reported (Shortt, Smith, Krishnan and Swaminath, 1931) Before reporting our results it will be as well to make a rapid survey of the previous experiments in India with this susceptible mammal

Shortt, Barraud and Craighead (1927) used 20 hamsters, of these four were not examined The remaining 16 were subjected to an average of 34 feeds each by supposedly infected sandflies, of which it was estimated that 9 were actually infected The first feed on each hamster was given between 12th February and 22nd May, so that one may assume that the majority of the feeds were given during the hot weather, a few being given during the comparatively cool month of February The period from the commencement of the experiment to the time of examination of the hamsters was from 3 to 6½ months, the average being 5½ months We assume, though it is not stated, that all the animals were sacrificed and that a satisfactory cultural examination was conducted in each case No transmissions were recorded in this series

Shorff, C. (1928), and Swaminath (1928) used 15 hamsters, of one was not infected, but one died and no culture was obtained, the others fed upon by a total of 7 sandflies each of which it was estimated that 5 infected flies could infect 10% of the hamsters were fed upon by an average of 240 flies of which 30% were infected. The animals were sacrificed and examined for flagellate infection. The date of the commencement of the experiment was from 25th January to 10th September of the year. The time of observation of the hamsters was from 1 to 14 months, average being 9 months, in 3 cases the period was over 1 year.

The other series of the one referred to above in which one hamster was infected, there were 7 hamsters, of these 5 died and no parasites were obtained. The remaining two hamsters had, respectively 14 and 19 flies fed on each, 22 and 19 were probably infected. The feeding commenced in the afternoon and ended into the cold weather. The times from the commencement of the experiments were 511 and 510 days respectively, infection was obtained in the former case.

In addition to these reported experiments between the years 1926 and 1928, about 12 hamsters have been used in similar infection experiments at the Calcutta School of Tropical Medicine. Dissections to see how many of such flies were infected were not made, as the staff available for this work was limited and other work at the time considered more important would have to have been sacrificed had we altered our methods to enable us to make the exact enumerations we have made in the present series. Later, these experiments were abandoned entirely in favour of human experiments. In no case were the hamsters maintained alive for more than a year from the beginning of the experiment. No transmission were obtained.

THE PRESENT SERIES OF EXPERIMENTS

The technique adopted was very much the same as that of the previous series. The sandflies used in the experiment were bred in the laboratory, they were fed on kala-azar patients in the wards of the hospital attached to the Calcutta School of Tropical Medicine, they were given a second feed on the same or another patient, the third and all subsequent feeds were given on hamsters, and finally when the flies died they were dissected. The flies were bred and kept in an incubator with a temperature of 28°C at the commencement of the experiment, but at a later date the temperature was raised to about 30°C as we found that during the monsoon months this was the temperature maintained steadily in the huts in the villages from which many of our patients had come. The flies were allowed to feed as many times as they would, and when they died they were dissected. The number of infected flies that were known to have fed on each hamster was noted, the proportion of the dissected flies showing a flagellate infection was also noted, the figure and the total number of flies that had fed on each hamster, the number of infected flies was calculated. The flies were fed on a shaved area of the abdomen of the hamster.

TABLE I

Serial number of hamster	BANDERLES FED ON THE HAMSTER			Season during which feeds were given	Days from first feed to liver puncture	Result of liver puncture	Duration of experiment from first feed to death of the hamster	Result of examination
	Total	Number actually infected	Probable number infected					
3	147	10	37	H and M	571	-	571	-
5	170	12	42	P and M	580	-	620	-
7	153	8	47	M	535	+	583	-
9	105	10	32	M	522	-	571	-
11	92	9	32	M	518	-	567	-
13	54	8	29	M	455	-	502	-
15	129	8	32	M and P M	154	-	500	-
17	179	17	56	M	167	-	517	-
19	101	12	34	M			438	-
21	100	11	42	P M			419	+
23	111	14	48	P M, C H			419	-
25	144	119	431				5,716	
27	144	11	30				520	

M = monsoon (June to September) P M = post monsoon period (October to mid November to February)

There were 32 hamsters involved in this experiment. In four cases none of the flies that fed on them was proved to be infected, no further reference will be made to these. The remaining hamsters can be divided readily into three groups, each group will be considered separately —

Group I — The 11 hamsters of this group survived until the end of the experiment and were killed between the 419th and the 629th day of the experiment, the average duration of the experiment was 520 days, the average number of flies fed on them was 144 of which 39 were probably infected. Smears were made from their spleens, livers and bone-marrow, and cultures were made from their spleens and livers, *in one case a leishmania culture was obtained from both spleen and liver*.

In 8 cases in this group liver puncture was done, in one of these a body which all the writers agreed was a typical leishmania parasite was found on the 435th day of the experiment, when, however, this animal was killed on the 583rd day no parasites were seen and the culture remained sterile.

Further details are given in Table I above.

Group II — The 9 hamsters of this group died during the course of the experiment after having had a comparatively large number of sandflies fed on them, the average duration of the experiments was 238 days (in three instances it was over 400 days), the average number of flies fed on each was 134 of which 33 were probably infected. Smears were made from the liver, spleen and bone-marrow, and in some cases cultures were attempted, but in every case these were contaminated as decomposition had set in. *Of this group one hamster had a heavy leishmania infection in all the smears examined*, this animal had died on the 482nd day of the experiment.

Further details are given in Table II below —

TABLE II

Serial number of hamster	SANDFLIES FED ON THE HAMSTER			Dates of the first and last feeds	Season during which feeds were given*	Duration of experiment from first feed to death of the hamster
	Total	Number actually infected	Probable number infected			
1	109	6	17	26-4-31-27-11-31	H M, P M	120
2	83	9	31	4-6-31-23-9-31	M	170
6	198	9	52	5-6-31-8-9-31	M	511
8	180	16	48	25-6-31-21-8-31	M	147
10	95	7	26	25-6-31-27-8-31	M	64
12	167	12	51	30-6-31-3-8-31	M	482
16	143	10	26	15-8-31-17-11-31	M and P M	404
25	127	5	20	31-8-31-22-11-31	M and P M	220
31	102	6	23	13-4-32-17-5-32	H	36
TOTALS	1,204	80	294			2,144
MEANS .	134	9	33			238

* See footnote to Table I

Group III—This consists of 8 hamsters that died in the earlier stage of the experiment when less than 5 proved-infected flies had been fed on them. The average duration of the experiment in this group was 80 days, the average number of flies fed on each was 48, of which 6 were probably infected. Smears from the organs of these animals were examined, but no satisfactory cultures were made. *No infections were demonstrated in this series.* Further details are given in Table III below —

TABLE III

Serial number of hamster	SANDFLIES FED ON THE HAMSTER			Duration of experiment from first feed to death of the hamster
	Total	Number actually infected	Probable number infected	
4	83	2	7	242
17	14	1	7	22
26	27	1	3	5
27	52	1	4	144
29	56	2	7	126
32	32	1	3	32
33	97	3	7	40
35	21	4	10	30
TOTALS	382	15	48	641
MEANS	48	2	6	80

COMPARISON OF THE EARLIER EXPERIMENTS WITH THE PRESENT SERIES

In the first series (1927) of experiments, most of the feeds were given during the hot weather, the number of flies fed on each hamster was comparatively small (mean=34) and in no case was the hamster allowed to survive more than $6\frac{1}{2}$ months, any one of these three points might be an excuse for the failure in this series.

The second series (1928) can be compared with our present series reported in Table I. It will be seen that in the former the number of flies fed on each hamster and the number of 'probably' infected flies was nearly twice that of our series. The season at which the two sets of experiments were carried out was comparable, but in our series they were both commenced and concluded at a later time of the year, and in our successful experiment the feedings were given at the end of the post-monsoon period when the weather was beginning to get cool again. However, the time the hamsters were allowed to live was definitely longer in our series, an average of 17 months against $14\frac{1}{2}$ months, furthermore in every instance the period was more than 400 days, whereas this length of duration was only achieved in three instances in the other series.

Let us consider the successful experiments, the hamsters survived 511, 482 and 419 days, and the seasons when they were fed upon by the infected flies were

the late monsoon, the early monsoon and the post-monsoon periods, respectively. In the experiment that we must class as doubtful, on account of our failure at a later date to confirm our finding by culture (though in this case it seems just possible that a very scanty infection died out), the period was 535 days and the flies were fed on the hamster during the early monsoon period.

Thus, the essential factors for successful transmission appear to be that the hamster shall survive for at least 400 days and that the feedings shall be given during a period when the atmospheric humidity is high. However, it is clear that these are not the only factors as in eleven other instances in the present series of experiments no transmission occurred despite these conditions being complied with.

Young, Hertig and Liu (1929) found that when a small dose of parasites is administered there is a very distinct prolongation of the incubation period, and further that in these cases there is a greater chance of spontaneous recovery on the part of the hamster.

The difficulties in carrying out this type of experiment are thus seen to be very great, if one keeps the hamster alive for the long period that appears to be necessary for development of the infection, it may die during the night and be decomposed by the morning, and, furthermore, an infection produced by a small dose of flagellates may die out, on the other hand, if one performs liver puncture at frequent intervals, one will most certainly lose the majority of the animals at a too early date.

SUMMARY

Amongst 28 Chinese hamsters fed upon by sandflies (*Phlebotomus argentipes*) that had previously fed upon kala-azar patients, two became definitely infected with *Leishmania donovani*, and one apparently had a transitory infection.

All these animals survived for more than 400 days from the commencement of the experiment, and the feedings were carried out during a period of the year when the atmospheric humidity was high.

ACKNOWLEDGMENTS

We wish to place on record our appreciation of the assistance rendered by the following members of the staff of the inquiry, Drs C R Das Gupta, S Mookerjee, Chianji Lal and L R Sharma, without whose active co-operation this work would not have been satisfactorily accomplished.

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THE RELATIVE INFECTIVITY OF THE TWO FORMS OF *LEISHMANIA DONOVANI* ADMINISTERED BY DIFFERENT ROUTES

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THE experiments reported here were undertaken to ascertain the relative susceptibility to infection by *Leishmania donovani* of the hamster (*Cricetulus griseus*) when the parasite is administered by different routes and at different stages of its life cycle

It was first shown by Smyly and Young (1924) that the Chinese hamster was easily infected by the administration of leishmania in the form of an emulsion made from spleen puncture material into the peritoneal cavity. Later Young, Smyly and Brown (1926) extended their observations and in a large series of experiments showed that when spleen emulsion from an infected hamster was injected into the peritoneal cavity of another hamster the parasite could be recovered from the liver in as short a period as four days, and that a generalized infection in the hamster persisted for 11½ months. They found that 2 out of 5 hamsters became infected when spleen emulsion was injected into the pleural cavity, and that 3 out of 14 became infected when it was given by the subcutaneous route. Their attempts to produce infection by oral administration failed, but in no instance did they allow the hamster to survive for more than 18 days, nor did they check their findings by cultural examination of the organs.

Hindle, Hou and Patton (1926) carried out a small series of experiments to ascertain the infection rates when the parasites were administered in the flagellate

and in the 'resting' stage, by different routes, they first checked their results by liver puncture and subsequently gave half the animals in each batch an intraperitoneal injection to demonstrate their susceptibility. As liver puncture is an uncertain method of demonstrating infectivity, this procedure vitiated about half their experiments, the results of the remaining experiments can be summarized as follows -

	SPLEEN EMULSION		FLAGELLATE CULTURES	
	Total	Infected	Total	Infected
Percutaneous	5	0	5	1
Subcutaneous	5	1	5	1

By the intraperitoneal method they produced infection in 10 out of 14 hamsters, using 12-day-old flagellate cultures. They sacrificed and examined their animals after a maximum of $3\frac{1}{2}$ months, and they did not check their results by culture as a routine procedure.

Shortt, Craighead, Smith and Swaminath (1928) infected one hamster by dropping spleen emulsion into its mouth and another by dropping this material on to its conjunctival membrane, only two hamsters were used in this experiment and they were allowed to survive 363 days before being killed. The same workers (1929) reported another series of infection experiments carried out by the workers of the Kala-azar Commission, they infected 8 out of 10 hamsters by a single oral administration of spleen emulsion in a 335-day experiment, and 3 out of 6 by repeated administrations, 88 in one case, of concentrated flagellate cultures, the times from the beginning of the experiment being between 373 and 406 days.

Young, Hertig and Lau (1929) reported over a thousand infection experiments with hamsters, most of these were by the injection of spleen or liver emulsion into the peritoneal cavity in different doses. They found that the most minute doses produced infection, but that where small doses were given a generalized infection took much longer to develop and tended to die out more readily. By percutaneous administration they produced 18 infections in 63 experiments using infected organs of hamsters as their source of material, and 7 out of 37 when they used flagellate cultures. Their attempts to produce an infection by the oral route still failed.

Khaw (1930) on the other hand produced an infection in 12 out of 14 hamsters by the oral administration of spleen emulsion.

We do not claim that this is in any way a complete review of the literature on the subject, but the more important experiments have been summarized. None of these workers had done any experiments primarily designed to compare the

infectivity of the parasite in its two different stages, the flagellate and the 'round', when administered by different routes

We felt that an experiment of this kind was necessary. After we had commenced this series of experiments Shortt and Swaminath (1932) published the results of a small series of comparative experiments on much the same lines as those we had planned, they, however, used only strong emulsions and concentrated cultures, and there were only 29 hamsters in their series.

All the hamsters that we used in these experiments are imported from China and a large percentage always die on the way, so we were compelled to be economical in our use of animals and, whereas we had hoped to have ten in each batch we had to limit some of the batches to five hamsters.

The opinion we had formed from our own experiments and from those of other workers was that the peritoneal route was the most successful with either form of the parasite, that the oral was probably the next, with the 'round' form at least, and that the subcutaneous, the conjunctival and the percutaneous followed in that order, also there seemed little doubt that the flagellate stage was less infective than the 'round' stage, and that with the former larger and repeated doses would be necessary. These preconceived opinions influenced our decision regarding the size of the dose and the number of animals used in each experiment, we used five hamsters in the group in which we thought infection was either certain, or very unlikely, whereas with those about which we felt uncertain we used ten hamsters. Were we to do the experiment again we should make many modifications, we should certainly give much smaller and only single doses throughout.

TECHNIQUE

The hamsters—Each hamster was kept in a separate cage which was labelled by means of a metal disc wired to the cage, on this disc the letters of the group to which the hamster belonged and its own number in that group were painted, in white letters on a black background in the flagellate series, and in red on a white background in the 'leishmania' series. Each group of hamsters was kept on a separate tray and during the process of administration of the doses only one tray was brought into the field of operation at one time. Except when they were given their doses, no hamster was ever taken out of its cage, the stale food was shaken through the bottom of the cage and fresh food added daily without the hamster being touched. (This food consisted of soaked *gram* and a small piece of green-stuff, usually cabbage). There was thus no chance whatsoever of hamsters becoming interchanged. If a hamster died during the experiment, he was usually replaced by another hamster which was then given the appropriate dose or doses of infecting material.

The flagellate cultures—These were first sub-cultures, 12 days old, on N N N medium. The 'strong' doses consisted of the condensation fluid from one tube of medium, and the 'dilute' doses of about 0.1 c.c. of diluted culture, estimated by direct counting in a haemocytometer chamber to contain 300,000 flagellates.

The 'leishmania' forms—These were obtained by killing an infected hamster and emulsifying the spleen and liver under aseptic conditions. Citrate saline was added to this emulsion which was then filtered through a coarse filter-paper. This

constituted the 'strong' emulsion. A rough estimate of the number of parasites per cubic centimetre was made with the aid of a counted pigeon's-blood suspension, and the emulsion was diluted with citrate saline to contain about 300,000 parasites per 0.1 c.c., for the 'dilute' emulsion.

The administration of the doses—The repeated doses were given at weekly intervals, ten doses being given in each case. The oral doses were given from a hypodermic syringe with a blunt needle, usually into the pouch of the hamster. The subcutaneous doses were given under the skin of the back, so there was no chance of accidental inoculation into the peritoneum. The percutaneous doses were given by strapping the hamster on its back, shaving the abdomen, scarifying with a hypodermic needle a small area on the shaved patch, gently spreading a drop of infecting material over the scarified area, and leaving the hamster tied up until the area was quite dry. We watched the hamsters to see if they attempted to lick the scarified area, but in no case was this observed. In the case of the repeated percutaneous doses, the four quadrants of the shaved area of the abdomen were scarified in turn. In the case of the conjunctival doses two or three drops were dropped on to the open eye at half-minute intervals.

The examination of the hamsters—In a few instances the hamsters died towards the end of the experiment and were not replaced. These were examined, smears were made from both spleen, liver and bone-marrow, and cultures were sometimes attempted, but they were contaminated in every instance. In the case of the groups which were expected to be 'positive' a preliminary liver puncture was done to obviate sacrificing the animals, if this were positive, the animal was not killed, but if it were negative the animal was sacrificed in the usual way and cultures made from its organs. The remainder of the hamsters were killed between six and eight months from the beginning of the experiment and the spleen and liver examined both directly and by cultural methods. The procedure we adopted was that described by Christophers, Shortt and Barnaud (1926), pieces of spleen and liver were emulsified separately and two N N N tubes inoculated from each. Only in the case of one of the sacrificed animals were the tubes contaminated, in those cases in which animals died all the tubes were contaminated.

The results of the two sets of experiments are given in the two tables below.

The degree of infection—There is no very satisfactory way of deciding on the degree of infection exhibited by the animals. We adopted a system of giving one, two, three or four *plus* signs in each case in which parasites were found in the smears, but the distinctions were necessarily arbitrary and it is doubtful if the method is of much value on this account. It is, however, possible to divide the infected hamsters into three classes, namely, those in which infection was demonstrable by liver puncture, those in which it was demonstrable in smears from the organs, and those in which it was demonstrable by cultural methods only. It is probable that these indicate three distinct degrees of infection. Unfortunately, liver puncture was not done in every case, in fact in the 'flagellate' series it was only done in five cases, in all of which it was 'positive'. In the other series it was done in 33 cases, of which 15 had received doses by the mouth, 15 by subcutaneous injection, and 3 by intraperitoneal injection, in these three groups 3, 7 and 2, respectively, were 'positive'. Or, to compare them more in detail, in the ORD and SRD groups 1 in 3 and 5 in 5 were positive, in the OSS and SSS groups 1 in 4 and 0 in 4 were positive, and in the OSD and SSD groups 0 in 6 and 2 in 6 were positive, respectively.

TABLE I

			Sacred simeas			Cultures		Died simeas		Proved positive	Proved negative	Ratio of proved positive to proved negative
			+	-		+	-	+	-			
Oral	Repeated	Strong	7	2	{ 18/9	2	0	0	1	9	{ 22/5	
		Dilute	6	2		0	2	0	2	6		
	Single	Strong	2	3		1	2	1		3		2
		Dilute	3	2		1	1	1		4		1
Subcutaneous	Repeated	Strong	3	0	{ 28/1			2	0	5	{ 33/1	
		Dilute	9	0				1	0	10		0
	Single	Strong	8	0				1	1	9		0
		Dilute	8	1		0	1	1	0	9		1
Per cutaneous	Repeated Single		4	1	{ 5/5	0	0*			4	{ 8/1	
			1	4		3	1			4		1
Intra-peritoneal	Strong Dilute		4	0	{ 9/0			1	0	5	{ 10/0	
			5	0						5		0
Conjunctival	Strong		0	9	{ 0/9			0	1	0	{ 0/9	
												9
	TOTALS		60	21		7	7	6	5	73	16	

* Cultures contaminated

TABLE II

SACRIFICED OR LIVER PUNCTURED				FOUND DEAD			Proved		Ratio of proved positive to proved negative	
Smears		Ratio of + / - smears	Cultures of positive smears		Smears					
+	-		+	-	+	-	+	-		
Oral	Repeated { Single {	Strong	{ 12/8 }	{ 0 1 2 6 }					17/7	
		Dilute								
		Strong								
		Dilute								
Subcutaneous	Repeated { Single {	Strong*	{ 11/5 }	{ 0 3 2 }					14/4	
		Dilute								
		Strong								
		Dilute								
Per cutaneous	Repeated Single		{ 2/6 }	{ 4 2 }	{ 2 1 }	{ 2 1 }			6/3	
Conjunctival	Repeated Single		{ 4/5 }	{ 1 1 }	{ 0 1 }	{ 1 3 }			5/4	
Intra peritoneal	Dilute		2/1	{ 1 }	{ 1 }	{ 0 }			3/0	
Totals				31	25	7	18	5	45	18

* Omitted as 100 per cent infections anticipated

The flagellate series—There were very few anomalous results in this series, that is to say in practically every group there were fewer negative results in the 'repeated' than in the 'single' and in the 'strong' there were fewer than in the 'dilute' sub-groups. Furthermore, in the 'intraperitoneal' group parasites were found in the spleen smears in every case.

Comparison between the different routes—The 'intraperitoneal' route is, of course, the most certain, we really introduced this group as a control. The conjunctival route is obviously the most uncertain, in no instance was infection produced in this group, but unfortunately only single doses were given, though these were of the undiluted flagellate culture. The subcutaneous is the next most certain route, it will be seen from column 10 of Table I that there were 33 'proved-positive' to one 'proved-negative' result, the only failure by this route being one in which a hamster was given a single dilute dose. In this group all the animals were heavily infected, as the 'negative' hamster was the only one in which parasites were not found in the smears. In the 'oral' group the infection rate was not so high, the 'proved-positives' to 'proved-negatives' being 22 to 5, and furthermore in 4 other hamsters the infection was not sufficiently heavy to be noted in smears. The 'percutaneous' group provided a larger number of 'proved-positive' results than we had been led to expect, but in half these the infection was only demonstrable by cultural methods, therefore, though the proportion of 'proved-positives' to 'proved-negatives' was greater in this group, it cannot be placed above the oral group. The various routes can be placed in the following order according to their potentialities in allowing the entry of the infecting organism in its flagellate stage. The peritoneal, the subcutaneous, the oral, the percutaneous and the conjunctival.

The 'round' or 'leishmania' series—In this series there are a few apparent anomalies. We reduced our intraperitoneal group to five and gave only diluted material. Unfortunately two of the animals died prematurely, in one case no parasites were found in the direct smear, but the infection was afterwards proved by culture. Again, we omitted the 'repeated strong subcutaneous' doses because we thought that they must inevitably all be 'positive'. In the 'subcutaneous single strong' sub-group there were 3 'proved-negative' to one 'proved-positive' finding, this is a surprising result, especially as in the group with the dilute material the results were reversed and there were 8 'proved-positive' to one 'proved-negative' results. The explanation may be that in the undiluted spleen emulsion there was a large amount of antibody present which prevented infection, but the more likely explanation appears to be that a sharp local reaction was set up and the parasites shut off and destroyed.

Comparison of the different routes—Our results with the intraperitoneal route were too few to stand alone as evidence of the superiority of this route, but, such as they were, they added confirmatory evidence to the well-established opinion that this is the route by which infection is most easily produced. In order to compare the subcutaneous with the oral group the 'repeated strong' sub-group should be excluded in the latter group but even if this sub-group is not excluded the proportion between the 'proved-positive' and the 'proved-negative' is distinctly greater in the subcutaneous group. Also, the liver puncture results, referred to above, indicate that the degree of infection was greater in the subcutaneous group, as in 7 out of 15 instances the parasite was found by liver puncture, against 3 out of 15 in the oral

group. However, the oral group gives definitely better results than the percutaneous. In this series the conjunctival route also provides a number of 'positives'; the results are not strictly comparable to those of the conjunctival group in the flagellate series, as in the 'leishmania' series we gave repeated doses in 5 cases. However, even with the single doses two positive results were obtained. The various routes can be placed in the following order according to their potentialities in allowing the entry of the parasite in its leishmania stage. The peritoneal, the subcutaneous, the oral, the percutaneous and the conjunctival.

Comparison between the infectivity of the flagellate form and the leishmania form—There are a few criticisms that could be made if we attempted to lay too much emphasis on the strict comparability of the results in these two series of experiments. The number of parasites of each phase given was of course not exactly the same. In the case of the dilute doses an attempt was made to bring the parasites to about the same figure, namely, 300,000, but the methods of estimation were necessarily rough. In the case of the strong doses no such attempt was made. There is no doubt that there were many times as many parasites in the strong emulsion as there were in the flagellate cultures, no attempt was made to concentrate the latter, as was done in the case of most of the earlier experiments we have referred to above.

Another point of difference was that the experiments were carried out at a different time of year, the first, the flagellate, series commenced in October and the other series in April, the reason for this was that we wanted the infected hamsters of our first series as the source of material for the second. It seems very unlikely that either the difference in the time of year, or the longer residence of the second batch of hamsters under laboratory conditions would in any way affect the results of the experiments.

Lastly, the medium in which the parasites were suspended prior to inoculation was different in the two sets of experiments, in one case there was a large amount of cellular debris from the emulsified spleens and livers, whereas in the other the parasite was suspended in citrate saline in which a little hæmoglobin was dissolved.

The presence of this foreign matter might have lowered the animals' powers of resistance to infection, on the other hand, the addition of so much antigenic matter might have increased them, passively.

With these reservations, a comparison between the two sets of figures in the two tables can be made.

Taking each series as a whole we see that in the flagellate series of the 89 cases in which a 'proved' result was obtained 73 or 82 per cent were 'positive', against 45 out of 63 or 71 per cent in the leishmania series, and in the sacrificed animals 71 per cent of the smears were positive in the former series against 55 per cent in the latter.

Comparing the corresponding routes in each set, we see that in the oral, subcutaneous and percutaneous groups the infection rate is higher in the flagellate series, whether the results are judged on the smears or on the cultural findings, but that in the conjunctival group the leishmania form appears to be infective whereas the flagellate does not, by the peritoneal route the results were consistently positive in whichever stage the parasite was injected.

As any numerical advantage that there was was in favour of the leishmania stage, we must conclude that the flagellate stage is the more infective stage by every route but the conjunctival

In view of the fact that there has been much speculation on the subject, that some work has been done to find out which is the 'infective' form of *Leishmania donovani*, and that various so-called 'post-flagellate', 'encysted', and 'granular' forms have been described and special powers of infectivity attributed to them, it is interesting that in the first large series of comparative experiments the results should point to the flagellate being the most infective form. We chose 12-day cultures because the results in a previous infection experiment with mice (Napier, 1927) indicated that there was little increase in the infectivity of an N N N culture after the 12th day. The earlier flagellate is quite possibly as infective, but younger cultures would contain fewer parasites. In the case of the conjunctival inoculations it seems probable that the parasites do not gain entry through this membrane, but are washed down the lacrymal ducts and gain entry somewhere in the intestinal canal, in the case of the flagellates the lacrymal fluid apparently killed them, whereas the intracellular leishmania form were able to survive.

Splenic enlargement—In every case the size of the spleen of the hamster was observed. Again, an arbitrary method of noting these observations was adopted, and they were marked as 'normal', 'enlarged', 'definitely enlarged', and 'enlarged two, three or more times'. For analysing our observations we have only included those cases in which the animal was sacrificed, and we have divided the enlarged spleens into 'definitely enlarged' and 'markedly enlarged', the latter group including all spleens enlarged three times or more. The results are shown below—

Size of spleen	Normal	? enlarged	Definitely enlarged	Markedly enlarged
Infected hamsters	10 (13.3 per cent)	15 (20 per cent)	15 (20 per cent)	35 (46.7 per cent)
Hamsters in which infection was only shown by cultural methods	5 (35.7 „)	5 (35.7 „)	2 (14.3 „)	2 (14.3 „)
Uninfected hamsters	24 (68.6 „)	6 (17.1 „)	4 (11.4 „)	1 (2.8 „)

It is obvious that there is a very definite correlation between splenic enlargement and infection, though this correlation is not complete, in 10 infected hamsters the spleen appeared to be normal, and in 5 in which it appeared to be enlarged no infection could be demonstrated. The fact that the cases in which infection is scanty, i.e. demonstrable only by cultural methods, show a degree of splenic enlargement less than the heavily infected but more than the uninfected cases does not suggest that these are instances in which the infection has been partially overcome by cellular response.

SUMMARY AND CONCLUSIONS

A series of comparative infection experiments have been carried out with *Leishmania donovani*, the causative organism of Indian kala-azar, in its flagellate stage as it is encountered in culture tubes, and in Nature in the sandfly, and in its 'round' or leishmania stage as it is encountered in the blood and organs of its vertebrate hosts

The susceptible vertebrate host used in the experiments was the Chinese hamster, *Cricetulus griseus*, and the parasite was administered by five different routes, the intraperitoneal, the subcutaneous, the oral, the percutaneous and the conjunctival

A high percentage of infections was produced by both forms of the parasite by all routes but the conjunctival, and by this route the leishmania form caused infection though the flagellate form did not

Except when administered by the conjunctival route, the flagellate stage of the parasite showed greater powers of infectivity than the leishmania stage

The intraperitoneal was the most certain route by which to bring about infection, the next in order was the subcutaneous, then the oral and the percutaneous and, lastly, the conjunctival

There was considerable correlation between splenic enlargement and the degree of the infection in the hamsters

The points of particular interest are —

That the hamster is an extremely susceptible animal as evidenced by the high percentage of infections produced by comparatively small single doses

That the flagellate form enters and establishes itself in the tissues of the host more readily than does the leishmania form

That the parasite readily enters through an abraded skin surface

That the infection rate in our series of experiments was higher than that of most other workers, this was probably because we kept our hamsters for longer periods before sacrificing them and because we confirmed our findings by cultural methods

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IS HALOMETRY RELIABLE? A STATISTICAL VIEW

BY

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DR MAPLESTONE of the School of Tropical Medicine has measured the diameters of a large number of blood cells both by P Jones' method as well as by the method of diffraction (halometry) with a view to test the reliability of the latter. He has sent me his observational data together with graphs and histograms which he has prepared for purposes of statistical analysis. In the following pages I have explained the method of statistical analysis as followed by me and have also given the results of my studies of his figures.

Data supplied by Dr Maplestone —

(1) Observations by P Jones' method on diameters of 500 red blood corpuscles of each of ten healthy persons (of these nine had to be left out for the sake of grouping thus delimiting the observations to 4,991 cells only), *vide* Table I, 1st series

(2) Observations by the halometer on blood of the above ten, consisting of averages of six readings taken on two slides for each person—10 in all—*vide* Table II, 2nd series, C

(3) Observations by the halometer on blood of the same ten based on readings taken on two slides for five and one slide only for the rest—altogether 90—*vide* Table II, 2nd series, D

(The specimens of blood all obtained from the same ten individuals and as nearly as possible at the same time)

P Jones' method—This consists in making a thin dried blood film on a glass-slide and actually measuring to two places of decimals in μ the length and breadth of each cell and taking the means. The mean diameter of 500 cells is taken to represent the mean diameter of the red cells for any specimen of blood.

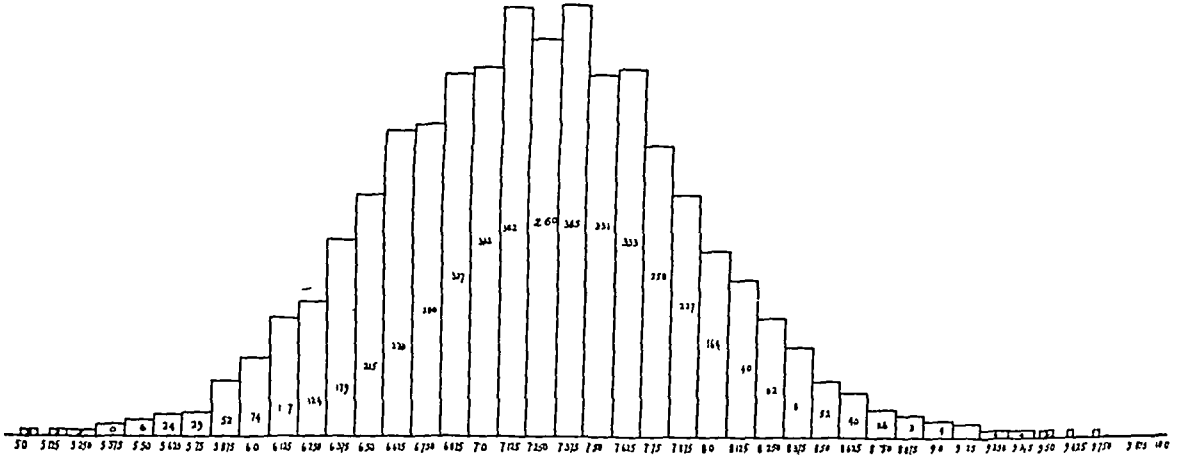
The halometer method—As described by Eve (1929) this consists in looking at the two coloured haloes inside the halometer, and moving these towards each other until their red edges just touch. A scale records the angular measurement and by reference to a printed table, the average size of the red cells can be obtained.

THE STATISTICAL PROCEDURE

The statistical analysis adopted by me consists of two parts The first part is concerned duectly with the inquiry in question (i e , reliability of halometry), and

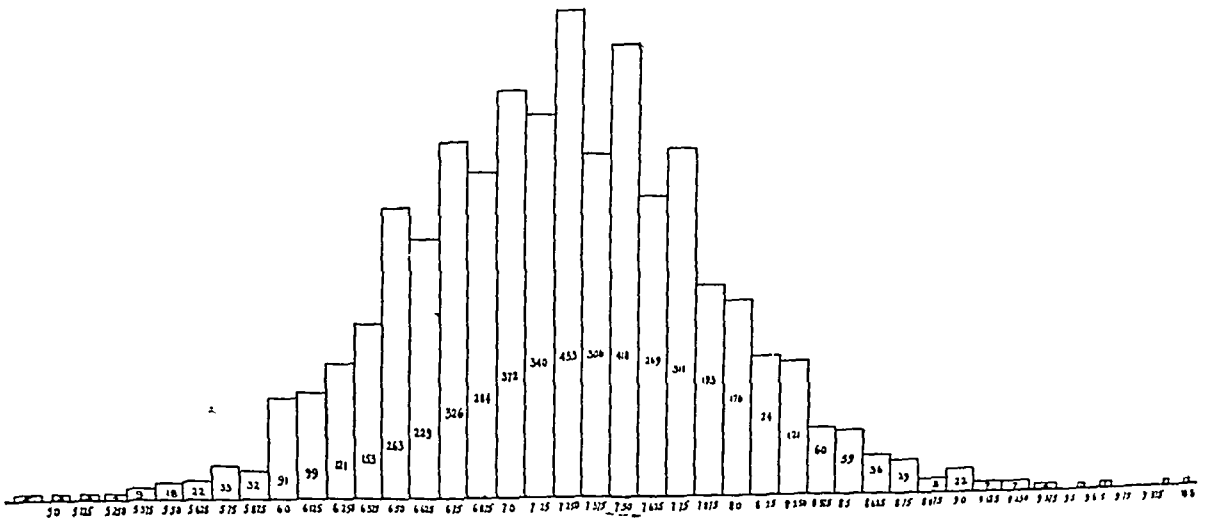
GRAPH 1

Histogram of 4,991 observations (grouped by 3)
(P JONES' METHOD)



GRAPH 2

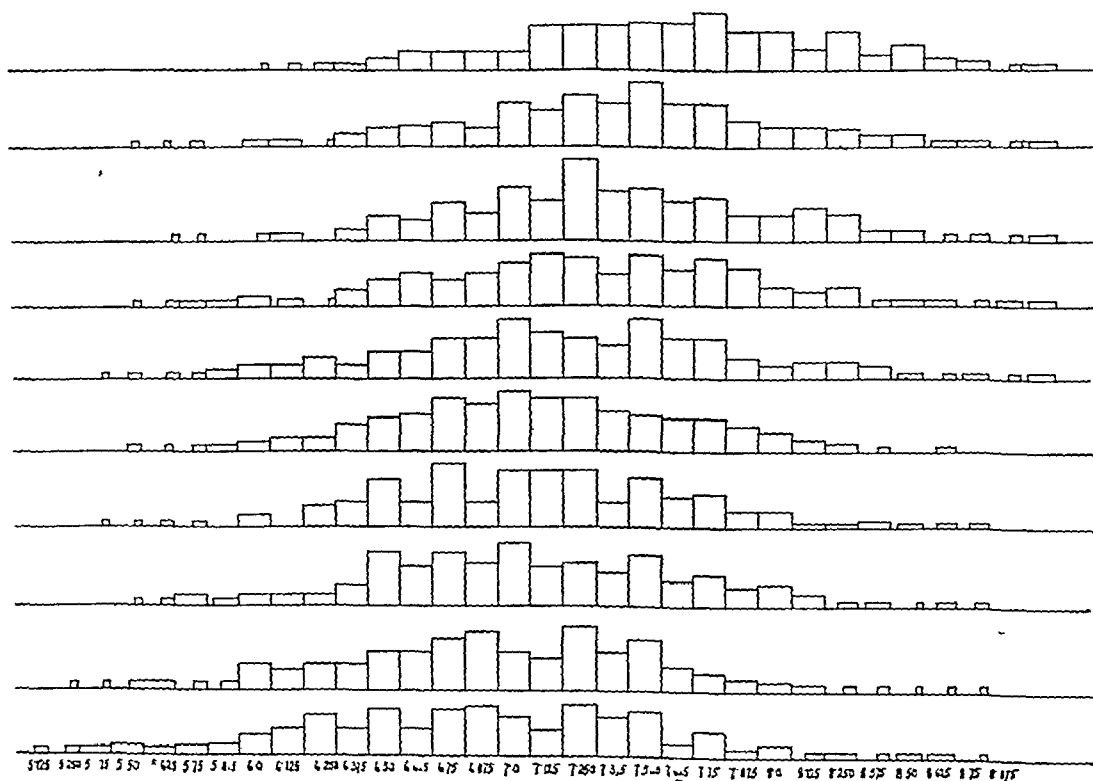
Histogram of 4,991 observations (by individuals)
(P JONES' METHOD)



the second part deals with the type of curve and the actual mathematical formula which fits best the blood cell population For either purpose it was found essential to rearrange the data in a suitable manner

Though the data have been supplied to me in carefully classified groups, such classification alone does not meet statistical needs nor bring out clearly the significance of the results. My object has been therefore to rearrange them in such a way that quantitative definition of the characters of the frequency distributions can be elicited and the corresponding characters of the two series may be quantitatively compared. The observations in each series have been rearranged as shown in Tables I and II. Tables III, IV and V contain my calculations. In Tables VI and VII is given the summary of the results obtained. Graphs 1 to 5 give the

GRAPH 3
Histograms of ten individual cases
(P JONES' METHOD)

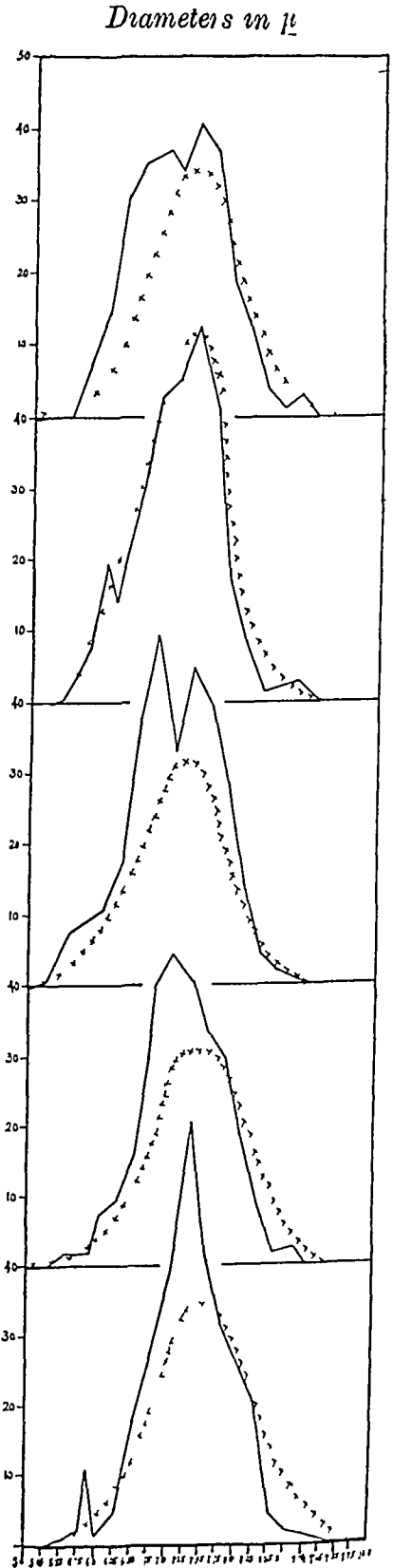
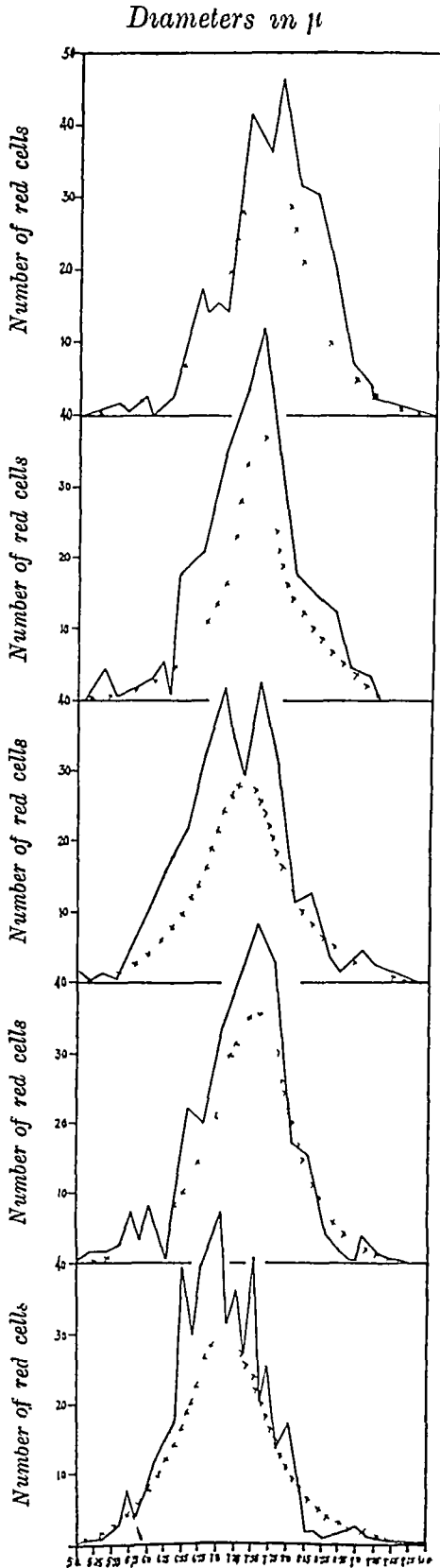


results in form of charts. From a perusal of these tables and charts the following will be evident —

The two parameters, mean $(\bar{x} = \frac{1}{n} \sum x)$ and standard deviation $(\sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{n-1}})$ have been calculated from the first two moments of the samples. As these two are especially related to normal distribution, these two alone are sufficient for summarizing the whole of the information as to distribution

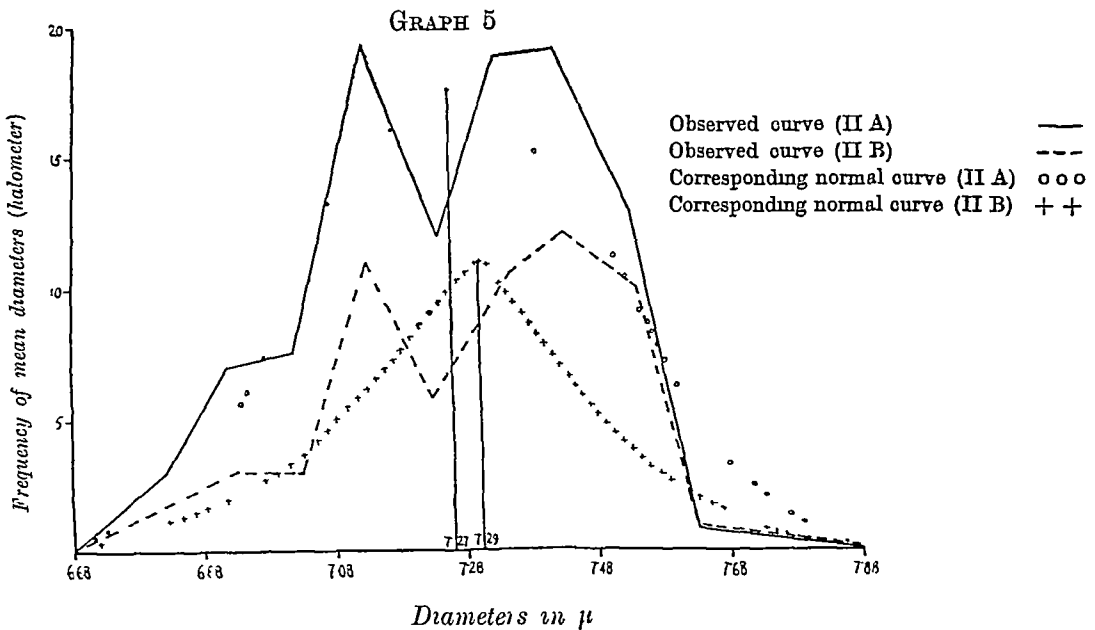
GRAPH 4

Frequency polygons of 10 individual cases
(P. JONES' METHOD)



which the sample provides. The calculation of the third and fourth moments would not have been necessary but for determining the type of curve as well as the formula that would fit it best. In order to attain this the measurements of diameters have been grouped together in equal intervals of the variate up to three decimal points and all the calculations have been carried out as shown in Tables III, IV and V. The working means have been selected about the central groups. Two corrections have been applied, one for the fact that the working mean differs from the true mean and the second because of grouping. The latter is known as Shephard's correction 0.125μ in the first series and 0.100μ in the second series have been selected as class units.

As the third and fourth moments by themselves are not sufficient to test the departures from normal distributions, from each of them two special functions, β_1 and β_2 , have been calculated, which in turn have given the values of a



quantity y_1 . This is 0 for a normal distribution and is distributed normally about 0 for a large sample. It is really a measure of asymmetry, while y_2 calculated from the fourth moment measures the type of departure from the normal form, by which the apex and the two tails of the curves are found at the expense of the intermediate portion.

Moreover, to bring out the significance of the results more distinctly two things have been done, first the errors of sampling have been estimated in each case and secondly a series of sixty observations have been chosen and its results compared with the results of 100 observations of the second series. Over and above the means have been compared, the normal type of distribution has been shown by means of polygons for each case, and the differences in variation amongst themselves have been brought out by means of histograms.

Having thus obtained the frequencies, the mean, the standard deviation and the type of curve, it will not be difficult to construct a curve. As regards the first series, the mathematical expression for curve being known, I have been able to evolve a formula which will fit the curve best. The theoretical curve which is calculated in this way is expected to correspond exactly with the curve of the observed data only if the whole of the infinite population could be measured. As that is, however, practically impossible, the large number (5,000) of cells has been measured, and we find that the actual and the expected curves do not tally exactly owing to the error of random sampling. But the only satisfactory test for the goodness of its fit is 'Chi-square' of Karl Pearson (1911) which has not been adopted here for want of space.

DISCUSSION OF RESULTS

Almost all the distributions of human attributes give normal curves, and it may be presumed that the red cells of the blood are no exception. The two principal characters in which such cases of normal distribution differ are its averages and dispersion, i.e., in the values of the variables round which they centre and in the range of variation. Over and above these fundamental characters the degree of asymmetry is of some value.

As regards the *averages* the value of the mean is not sufficient though it may serve for general purposes. The reason is that the *mean* does not represent the *mode* in case of asymmetrical distribution and that its value might be consequently misleading. They are not identical in our second series. For the same reason the value of the *median* has been estimated. Though the calculation of the mean alone is sufficient for the first part of our inquiry, it is not enough for finding out the type of curve or for testing the departure from normal distribution.

As regards the range of *dispersion* the simplest measure of the dispersion of a series of values of a variable is the actual *range*, i.e., the difference between the greatest and the least value observed. There are three such common measures, but *standard deviation* being the commonest and the best we have calculated that as well as the range of dispersion. In the first series it is 5μ to 10μ because it is a distribution of the original population of cells, while the range is 7μ to 8μ in the second series because it is a distribution of means of such samples. Similarly, the standard deviation is higher in the first series than that of the second. The reason is the fewness of the number as well as the limitation of the variation. The *coefficient of variation* ($c.v.$) calculated is merely the relative variability of the frequency distribution.

The parameters, however, give the characters of the population. Thus, from the various moments the two special functions, β_1 and β_2 , as well as from y_1 and y_2 , we have found that the first series of distributions represents a normal type, while the second series does not. Thus, with an ordinate at twice its standard deviation or three times the probable error, 98 per cent of cases lie within the limits in the first series. The skewness, the distance from the mode, the kurtosis and the criterion clearly indicate that the type of curve of the second is not normal. From further analysis and comparison of the two series, II(A) and II(B), we can clearly see that though it is not exactly normal, it has a tendency to be so as the number of frequencies are increased. Fisher (1925) says that if a quantity be normally distributed with standard deviation δ then

the means of random samples of n such quantities are normally distributed with standard deviation $\frac{\delta}{\sqrt{n}}$. Even when the original distributions were not exactly normal, that of the means usually tends to normality as the size of the sample increases. Now, given any frequency of a variate, the frequency of random sample of the means of 50 or 100 such variates is well known. If β_1 and β_2 are fundamental frequency constants of the primitive frequency then $B_1 = \frac{\beta_2}{3}$ and $B_2 - 3 = \frac{\beta_1 - 3}{n}$ are the fundamental frequency constants of the derived frequency of the means of n variates at a time, such means being calculated out of this primitive population. Thus, the mean of the means is the mean of the primitive population and the S.D., the only other constant needed, is $\frac{\delta}{\sqrt{n}}$. The population of the means should thus tend to become rapidly Gaussian. Now, Greenwood (1909) in a similar case has shown that the means of 25, 50 or 100 leucocytes, as obtained from the slide population, do not follow this rule but give far more skew distribution. Apart from these considerations, factors such as personal error and the functional error and the error of sampling, have to be considered before definite opinion on the significance of the results can be expressed. The readings taken by different observers, the technique of preparing the slides, and the method of calculating may all contribute to the cause of the deviation from the normal.

As the distributions have been taken from similar materials, we should expect that they should be in general of similar form but if they do not, then we may presume that these must be due to some defect in the apparatus or in the technique.

CONCLUSIONS

Measurements of 5,000 red blood corpuscles have been considered in the preceding pages and these had been taken from ten healthy persons almost about the same time, when (a) the first series consists of 5,000 observations by P. Jones' method, (b) the second *A* series consists of 100 observations on the means by the halometer and (c) *II B* series consists of 60 observations of the means by the same method. I find that —

- (1) The type of curve found in the first series is normal and the best fitting

curve is $Y = 368.714 e^{-\frac{x^2}{2(29.166)}}$

- (2) The curves of 500 cells of each of the ten individual cases show satisfactory goodness of fit with their respective normal curves

- (3) The curve of the *II A* series is not of normal type but it tends towards normality as the number in the sample increases

- (4) There is a difference in the curvature of the first series if taken individually and in groups of three each

- (5) There is a difference in the curvature between the first and the second series. Such result is consonant with the 'working errors' due to the variation of random sampling over and above the differentiation resulting from defective technique or from individual peculiarities in measuring and recording

- (6) The means of the first and second series are not significantly different

It will be evident from the above that there is hardly any difference between the results of the two methods for measuring the average diameters of red blood corpuscles. The halometer can therefore be safely relied on.

ACKNOWLEDGMENT

My thanks are due to Dr. Maplestone for giving me this opportunity of discussing the observations made by him with the halometer and thereby enabling me to further establish the value of this test by statistical method.

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APPENDIX

OBSERVATIONS

TABLE I
P. Jones' method
 (1st series)

Diameters in μ	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	Total	Regrouping by three
4 500—	2	1	2								5	
5 000—	1	1	1								3	3
5 125—	1	2									3	3
5 250—	1	1	2								4	5
5 375—	1	1	1	6							9	10
5 500—	1	1	2	1	2	1	2	8			18	16
5 625—	3	2	2	1	1	1	2	5	5		22	24
5 750—	8	6	2	2	1	2	2	2	8		33	29
5 875—	4	5	8	1	3	11					32	52
6 000—	9	9	12	4	1	2	8	11	20	15	91	74
6 125—	12	4	11	6	2	5	10	10	17	22	99	107
6 250—	15	1	17	1	3	5	10	18	20	31	121	124
6 375—	18	13	11	11	6	8	21	22	22	21	153	179
6 500—	40	23	22	18	10	18	27	38	31	36	263	215
6 625—	29	25	25	19	18	15	27	21	31	19	229	273

TABLE I—concl'd

Diameters in μ	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	Total	Regrouping by three
6 750—	40	21	33	21	15	28	40	50	43	35	326	280
6 875—	32	25	34	17	16	19	37	22	45	37	284	327
7 000—	48	34	42	36	15	40	45	34	44	34	372	332
7 125—	32	40	35	31	35	37	39	43	27	21	340	382
7 250—	37	39	30	44	42	61	41	45	53	41	433	360
7 375—	28	28	29	39	35	39	32	19	24	33	306	385
7 500—	41	49	43	52	37	42	34	40	42	38	418	331
7 625—	21	28	30	35	39	27	30	24	23	12	269	333
7 750—	26	43	31	35	47	32	30	29	18	20	311	258
7 875—	14	31	16	24	31	20	22	15	12	8	193	227
8 000—	18	18	12	18	32	21	19	15	9	14	176	164
8 125—	10	12	14	19	17	29	9	5	6	3	124	140
8 250—	3	17	13	16	31	21	8	5	2	5	121	102
8 375—	4	3	9	12	12	7	2	7	2	2	60	80
8 500—	1	5	4	13	21	6	4	1	4		59	52
8 625—	3	5	2	4	9	2	3	3	1	4	36	40
8 750—	2	2	4	6	7	3	3	1	1		29	26
8 875—	1	4	2	2	2	2					13	18
9 000—	4	4	5	5	4						22	14
9 125—	3	4									7	12
9 250—	1	2	3	1							7	6
9 375—	1	3									4	4
9 500—	1										1	2
9 625—	2										2	1
9 750—	0										0	1
9 875—	1										1	
10 000—	1										1	
TOTALS	520	512	509	499	495	504	507	493	510	451	5,000	4,991*

* Loss of nine frequencies owing to discarding of the fractional part in regrouping

TABLE II
P. Jones' method
 (2nd series)

Diameters in μ	A - C + D	B	C	D
6.68—6.77	1			1
6.78—6.87	3	2		3
6.88—6.97	7	3	1	6
6.98—7.07	7	3	1	6
7.08—7.17	10	11	2	17
7.18—7.27	12	6	1	11
7.28—7.37	18	11	2	16
7.38—7.47	18	12	2	16
7.48—7.57	13	10	1	12
7.58—7.67	1	1		1
7.68—7.77	1	1		1
TOTALS	100	60	10	90

CALCULATIONS

TABLE III
Calculation of variations
 (1st series)

Diameters in μ	Frequency (f)	Deviations from origin (x)	Frequency \ Deviation (fx)	Frequency (Deviation) ² (fx ²)	Frequency (Deviation) ³ (fx ³)	Frequency (Deviation) ⁴ (fx ⁴)
5.000—5.124	3	19	57	1,083	20,577	390,963
5.125—5.249	3	18	54	972	17,496	314,928
5.250—5.374	5	-17	-85	1,445	-24,565	417,605
5.375—5.499	10	16	160	2,560	40,960	655,360
5.500—5.624	15	15	240	3,600	54,000	810,000
5.625—5.749	24	14	336	4,704	65,856	921,984
5.750—5.874	20	13	260	3,380	43,713	578,260
5.875—5.999	32	-12	-384	4,608	-55,296	663,552
6.000—6.124	74	11	814	8,954	98,194	1,083,134
6.125—6.249	107	-10	-1,070	10,700	-107,000	1,070,000

TABLE III—contd

Diameters in μ	Frequency (f)	Deviations from origin (v)	Frequency × Deviation fx	Frequency × (Deviation) ² fx^2	Frequency × (Deviation) ³ fx^3	Frequency × (Deviation) ⁴ fx^4
6 250—6 374	124	— 9	1,116	10,044	90,396	813,564
6 375—6 499	179	— 8	1,432	11,456	91,648	733,184
6 500—6 624	215	— 7	1,505	10,535	73,745	516,215
6 625—6 749	273	— 6	1,638	9,828	58,968	353,808
6 750—6 874	280	— 5	1,400	7,000	35,000	175,000
6 875—6 999	327	— 4	1,308	5,232	20,920	83,712
7 000—7 124	332	— 3	996	2,988	8,964	26,892
7 125—7 249	382	— 2	764	1,528	3,056	6,112
7 250—7 374	360	— 1	360	360	360	360
7 375—7 499	385	0	0	0	0	0
7 500—7 624	331	+ 1	331	331	331	331
7 625—7 749	333	+ 2	666	1,332	2,664	5,328
7 750—7 874	258	+ 3	774	2,322	6,966	20,898
7 875—7 999	227	+ 4	908	3,632	14,528	58,112
8 000—8 124	164	+ 5	820	4,100	20,500	102,500
8 125—8 249	140	+ 6	840	5,040	30,240	181,440
8 250—8 374	102	+ 7	714	4,998	34,986	244,902
8 375—8 499	80	+ 8	640	5,120	40,960	327,680
8 500—8 624	52	+ 9	468	4,212	37,908	341,172
8 625—8 749	40	+ 10	400	4,000	40,000	400,000
8 750—8 874	26	+ 11	286	3,146	34,606	380,666
8 875—8 999	18	+ 12	216	2,592	31,104	373,248
9 000—9 124	14	+ 13	182	2,366	30,758	399,854
9 125—9 249	12	+ 14	168	2,352	32,928	460,992
9 250—9 374	6	+ 15	90	1,350	20,250	303,750
9 375—9 499	4	+ 16	64	1,024	16,384	262,144
9 500—9 624	2	+ 17	34	578	9,826	167,012

TABLE III—concl'd

Diameters in μ	Frequency (f)	Deviations from origin (x)	Frequency + Deviation fx	Frequency + (Deviation) ² fx^2	Frequency + (Deviation) ³ fx^3	Frequency + (Deviation) ⁴ fx^4
9 625—9 749	1	+18	18	324	5,832	104,976
9 750—9 874	1	+19	19	361	6,859	130,321
TOTALS	4,991		-6,698	154,558	-547,944	14,545,018

Moments about the arbitrary origin 7 437 μ

$$\nu_1 = -1\,34201$$

$$\nu_2 = +30\,967$$

$$\nu_3 = -109\,786$$

$$\nu_4 = +2,914\,24$$

Moments about the actual mean

$$\pi_1 = 0$$

$$\pi_2 = \nu_2 - \nu_1^2 = 30\,967 - (-1\,3420)^2 = 29\,166$$

$$\pi_3 = \nu_3 - 3\,\nu_1\,\nu_2 + 2\,\nu_1^3 = -109\,786 - 3(-1\,3420)(30\,967) + 2(-1\,3420)^3 = 10\,052$$

$$\pi_4 = \nu_4 - 4\,\nu_1\,\nu_3 + 6\,\nu_1^2\,\nu_2 - 3\,\nu_1^4 = 2,914\,24 - 4(-1\,342)(-109\,786) + 6(-1\,342)^2(30\,967) - 3(-1\,342)^4 = 2,649\,818$$

Shepherd's corrections are —

$$\mu_1 = 0$$

$$\mu_2 = \pi_2 - 0.08333 = 29.083$$

$$\mu_3 = \pi_3 = 10.052$$

$$\mu_4 = \pi_4 - \frac{1}{2}\pi_2 + 0.29167 = 2,649.818 - 14.583 + 0.29167 = 2,635.264$$

Besides the moments, two simple functions

$$\beta_1 = \frac{\mu_3^2}{\mu_2^3} = \frac{(10.052)^2}{(29.083)^3} = 0.0041705$$

$$\beta_2 = \frac{\mu_4}{\mu_2^2} = \frac{2,635.264}{(29.083)^2} = 3.1156$$

Two measures of symmetry =

$$y_1 = \pm\sqrt{\beta_1} = \pm 0.0645$$

$$y_2 = \beta_2 - 3 = +0.1156$$

$$\text{Skewness} = 0.029399 \pm 0.01168$$

$$\text{Criterion} = 0.0014092$$

$$\text{Type of curve, } Y = Y_0 e^{-\frac{x^2}{2\sigma^2}}$$

$$\text{Best fitting curve} - \gamma = 368.714 e^{-\frac{x^2}{2(29.166)}}$$

$$d = 0.0198 \pm 0.0073 \text{ (not significant) (distant from the mean)}$$

Mean	-7 270 μ \pm 0 0063
Mode	-7 289 μ
Median	-7 270 μ \pm 0 00791
S D	-0 674
C V	-9 270
Skewness	-0 029399 \pm 0 01168

TABLE IV
Calculation of variations
(II A series)

Average diameters in μ	f (Frequency)	z Deviation from origin (in class units)	fx Frequency \times Deviation	fx^2 Frequency \times (Deviation) ²	fx^3 Frequency \times (Deviation) ³	fx^4 Frequency \times (Deviation) ⁴
6.63—6.77	1	-6	-6	-36	-216	+1,296
6.75—6.87	3	-5	-15	-75	-375	-1,875
6.88—6.97	7	-4	-28	-112	-448	+1,792
6.98—7.07	7	-3	-21	-63	-189	+567
7.08—7.17	19	-2	-38	+76	-152	+304
7.18—7.27	12	-1	-12	+12	-12	+12
7.28—7.37	18	0	0	0	0	0
7.38—7.47	18	+1	+18	+18	+18	+18
7.48—7.57	13	+2	+26	+52	+104	+208
7.58—7.67	1	+3	+3	+9	+27	+81
7.68—7.77	1	+4	+4	+16	+64	+256
	100		-69	+469	-1,179	+6,409

Moments about the arbitrary origin 7.33 μ

$$v_1 = -0.69$$

$$v_2 = -4.69$$

$$v_3 = -11.79$$

$$v_4 = -64.09$$

Moments about the actual mean

$$\pi_1 = 0$$

$$\pi_2 = v_2 - v_1 = 4.69 - 0.4761 = 4.2139$$

$$\pi_3 = v_3 - 3 v_1 v_2 - 2 v_1^3 = -11.79 - 3(-0.69)(4.69) - 2(-0.69)^3 = -2.7387$$

$$\pi_4 = v_4 - 4 v_1 v_2^2 - 6 v_1^2 v_2 - 3 v_1^4 = 64.09 - 4(-0.69)(-11.79) + 6(-0.69)^2(4.69) - 3(-0.69)^4 = 44.2670$$

To the values of the moments given above certain corrections are necessary using μ to designate a corrected moment about the mean as origin

Shepherd's corrections are —

$$\mu'_1 = 0$$

$$\mu'_2 = \pi_2 - 0.03333 = 4.1306$$

$$\mu'_3 = \pi_3 - 2.7387$$

$$\mu'_4 = \pi_4 - \frac{1}{2} \pi_2^2 + 0.29167 = 42.4517$$

Besides the moments two simple functions

$$\text{Skewness} = -0.31909 \pm 0.0323 \quad \text{Distant of mode from the mean} = 0.065 \pm 0.0167$$

$$\text{Criterion} = -0.2203$$

$$\text{Type of curve, } Y = Y_0 e^{-\frac{x^2}{2\sigma^2}}$$

Mean	-7.261 $\mu \pm 0.136$
Mode	-7.326 μ
Median	-7.316 $\mu \pm 0.172$
S. D.	-0.2032
C. V.	-2.798
Skewness	-0.31909 ± 0.0323

OBSERVATIONS ON THE MODE OF ACTION OF QUININE IN MALARIA

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THE views regarding the mode of action of quinine in malaria are still very varied. While some think that the drug attacks the malarial parasites directly others consider that it does so in an indirect manner by stimulating the natural processes of immunity. In a previous paper, the writer (*vide* Krishnan, Lal and Napier, 1933), presented evidence that lent support to the latter view. Since doing that he has had several opportunities to study the question further in monkey malaria (*Plasmodium mui*?)* in great detail and to obtain more conclusive evidence in elucidation of this question. By the adoption of the supra-vital staining technique as a routine for the study of blood cells it has been possible not only to recognize, with a high degree of accuracy, the two types of phagocytic large mononuclear cells composing the reticulo-endothelial system (monocytes and histiocytes), but also to form a correct idea regarding the functional rôle of these cells in malarial immunity. Taking the evidence as a whole there appears to be little doubt that several factors contribute towards cure in malaria, but that among these the one that plays the predominant part is the capacity of the cells of the reticulo-endothelial system to respond to the stimulus of infection by mobilization, proliferation and functional activation. Administration of quinine merely heightens these responses and when they are adequate the disease is overcome, otherwise not. Factors such as the direct action of the drug on the parasite and infected red cells, as well as biochemical and other alterations in the serum, help to augment the efficiency of the phagocytic mechanism to varying extents. The writer has presented below the data collected by him on which these conclusions are based and it will be found that they lend support to his view.

* The strain of *Plasmodium* used, is the one discovered by Napier and Campbell in 1932 in a *M. cynomolgus* and later described by Knowles and Das Gupta (1932), and reviewed by Sinton and Mulligan (1932a).

332 *Observations on the Mode of Action of Quinine in Malaria*

Mobilization of large mononuclear cells -- That quinine in malaria is capable of mobilizing the phagocytic large mononuclear cells (monocytes and histiocytes) in the peripheral circulation and that this mobilization is closely correlated with clinical cure have been inferred from a study of the quantitative changes in the large mononuclear cells in 46 malarial monkeys, before and after the administration of the drug. Within 24 to 72 hours of administration of quinine there generally occurred a significant increase in the number of both types of large mononuclear cells and this increase was even more marked and rapid if the mode of administration was by injection instead of by mouth. In Table I a summary of the results of

TABLE I
SUMMARY OF RESULTS
(Vide Table II in the Appendix for details)

Species of monkey	Experiment	Number of observations	Average increase per cent of large mononuclears	Range of 98 per cent of cases
<i>M. rhesus</i>	Prior to recovery after quinine	44	73.53 ± 2.89	82.23 to 64.86
<i>M. rhesus</i>	Prior to natural cure	5	280.10 ± 5.04	295.22 to 264.98
<i>M. cynomolgus</i>	Prior to recovery after quinine	18	150.23 ± 14.73	203.09 to 97.37
<i>M. cynomolgus</i>	Prior to natural cure	3	217.33 ± 5.73	234.52 to 200.14
<i>M. rhesus</i>	No improvement after quinine (died)	12	(decreased) -21.94 ± 5.36	-5.86 to -38.02

82 observations is presented. It will be seen from the above table that in every case that showed subsequent clinical improvement the drug caused 75 to 280 per cent rise in the total phagocytic mononuclear cells and that when the drug failed to bring about the required degree of mobilization of these cells there invariably resulted no improvement in the clinical condition. If the effect of quinine in the two types of monkeys, *M. rhesus* and *M. cynomolgus*, is compared, it is found that in the *rhesus*, which is relatively more susceptible to malaria and which shows a heavier infection, there is generally a poorer mononuclear response to begin with and treatment, if instituted at the proper time, is followed by a significant increase in this response. On the other hand in the *cynomolgus* monkey which is relatively more resistant and shows a milder infection there is a fair degree of mobilization of large mononuclear cells (especially monocytes) even in the absence of quinine, and when the drug is administered it causes a further increase in the cellular response. In both species there appears to be a natural limit to the intensity of this cellular response and irrespective of whether quinine is given or not the highest count obtained prior

to cure in an animal is never above 30 or 35 per cent of the total white cells. This figure is more often reached in *cynomolgus* than in *rhesus* and it is invariably followed by cure or latency. In order to clinch the importance of this finding the writer left five heavily infected *rhesus* monkeys and three *cynomolgus* monkeys that happened to show a good cellular response untreated. It was found on subsequent examination that parasites were absent in the peripheral blood of every one of these animals. This, to the writer, is strong evidence of the efficacy of a good cellular response. It can therefore be safely asserted that there appears to be little doubt that mobilization of large mononuclear cells in the peripheral circulation is a phenomenon associated with recovery from malaria and that one of the actions of quinine is to intensify this mobilization, and to lead to the rapid engulfment and effective destruction of malarial parasites by the mobilized cells.

Proliferation of large mononuclear cells—That administration of quinine to animals suffering from malaria causes stimulation to proliferation of the large mononuclear cells (monocytes) has been inferred from the presence of increased numbers of dividing forms of monocytes* after the commencement of treatment. In the *rhesus* monkey that suffers from an acute and rapidly fatal infection these dividing forms are not as common as in the *cynomolgus* monkey which suffers from a chronic low grade relapsing type of infection. In both species of monkey, however, after administration of quinine a distinct increase in the number of these dividing forms is noticeable. When such an increase does take place the writer has noticed that it invariably signifies a good prognosis. These cells are seen whenever the animals are progressing towards recovery or latency, or whenever the number of parasites in them is being limited to a minimum or a low-grade persistent infection is present. In association with the increase of dividing forms one frequently notices a significant increase in the number of lymphocytes. Thus the writer is inclined to interpret as suggesting that lymphoid tissue is being stimulated and that the monocytes seen are probably of lymphatic origin†. If this explanation is correct then it certainly lends support to the view previously expressed by the writer that in pathological conditions at least, where there is a great demand for phagocytic cells, suitable stimuli may cause monocytes to originate from lymphatic tissue. The increased output of dividing monocytes as well as of monocytes of markedly different sizes into the peripheral circulation after quinine administration suggests that the drug intensifies the stimulation to proliferation of these cells. The initial stimulus that starts this process is the infecting agent and not quinine, the latter merely accelerates the process once it has been started. This stimulant action of the drug is an important factor in overcoming the malarial infection because it has been shown previously (*vide* Krishnan, Lal and Napier, 1932) that the function of monocytes and intermediate forms (stimulated monocytes) is to engulf and destroy young malarial parasites (merozoites) and therefore, when these

* The writer has identified these cells showing division of nucleus which have also been described by other workers (Cannon and Falaferro, 1931) as monocytes because firstly they are phagocytic and segregate neutral red in the form of a rosette of fine granules between the two nuclei and secondly, in association with their presence there is frequently an increase in the number of monocytes as well as a great variation in size of those present.

† *Vide* Krishnan, Lal and Napier (1932) for a detailed discussion.

cells are present in large numbers they will prevent the entry of parasites into red cells and check the intensity of infection

Functional activation of large mononuclear cells — That the presence of quinine in the circulating blood of malarial monkeys increases the powers of phagocytosis of the large mononuclear cells and possibly also their powers of destruction of the malarial parasites has been inferred from the following observations. After the administration of quinine the histiocytes are seen to contain larger numbers of infected red cells and parasites showing undoubted evidence of disintegration and digestion as well as increased amount of neutral red than prior to the use of the drug. Some of these cells can be seen with as many as ten or twelve infected red cells giving one the impression that they engulf infected red cells, *en masse*, after the administration of quinine (*vide* Plate, Krishnan, Lal and Napier, 1932). From this there is no doubt that the histiocytes play an important part in the removal of infected red cells from the circulation and therefore functional activation of these would mean a marked reduction in the number of developing forms of the parasites and an effective check on the schizogony cycle. Again, the monocytes which normally are poor phagocytes show increased powers of engulfment and contain a greater amount of pigment, debris, and neutral red. If our presumption that the intermediate forms are stimulated monocytes is correct, the increased numbers of these after quinine also mean functional activation of monocytes. It has already been stated that the function of monocytes and intermediate forms is phagocytosis and destruction of *free* malarial parasites. If this is accepted, then functional activation of these cells means an effective destruction of merozoites and a successful prevention of infection of other red cells. From this it will be clear that quinine increases the functional activity of the large mononuclear cells and that this action of the drug is an important factor in cure. Here it may be added that from certain observations made by the writer he is led to believe that death of protozoal parasites within phagocytic cells need not necessarily be due to any inherent destructive capacity of the cells themselves but that death of these may be brought about by the environmental conditions within the cells being unfavourable for their continued existence.

Increased susceptibility of parasites to phagocytosis by direct action — It has been suggested by some that quinine cures malaria by its direct lethal action on malarial parasites. From the numerous observations made the writer is led to presume that the beneficial results of the drug in malaria cannot be accounted for wholly by this effect. For, as with some human cases, in a certain percentage of malarial monkeys even enormous doses of quinine fail to bring about a cure, and in some others cure takes place after minute doses as well as in the complete absence of quinine. Therefore before formulating any theory regarding the mode of action of quinine one has to take into consideration these facts as well. The writer has already shown that there is a very close similarity between the cytological changes taking place prior to natural cure and those occurring prior to cure by quinine and also that, when quinine fails to bring about the required cytological change no clinical improvement results. From these he feels justified in saying that the chief way in which quinine acts is by accelerating the natural processes of cellular immunity. Although it is chiefly through them that quinine acts, there is no reason to disbelieve that the drug also helps by preparing parasites and infected red cells for phagocytosis and destruction by direct action. Chopra and Chowdhury (1929) have shown that when quinine

is circulating in the blood it causes a lowering of surface tension, as a result there will be an increase in the concentration of the drug at the various cell interfaces (Gibbs Thompson law). The degree of this concentration will depend upon the electrical condition at the interfaces and the effect of the concentration upon the nature of the cell concerned. If, at the time of the highest concentration of quinine in the blood, sporulation occurs, a certain amount of the drug (the positive alkaloid ions) will be adsorbed by the free malarial parasites (which are negatively charged). As a result there will occur an alteration in the electrical condition of the parasites which in turn may lead to their death or damage or increased susceptibility to phagocytosis. Of these three the last appears the most frequent and probable. If so, it will lead to the rapid engulfment and effective destruction of the parasites *only* if the cellular mechanism is functioning properly at the time. As regards the effect of quinine on infected red cells it will be somewhat similar to that on free parasites. On account of the negative charge the red cells carry, the positive alkaloid ions will be adsorbed by them as well, with the result that they become suitable for engulfment by histiocytes (*vide* Chopra and Chowdhury, 1932). Once they are within these phagocytic cells they get lysed and the parasites in them destroyed. Thus, while the monocytes deal with merozoites the histiocytes deal with infected red cells, jointly they bring about cure. The direct action of quinine therefore is to render parasites and infected red cells more susceptible to phagocytosis, the work of destruction in all probability being performed by and completed within the reticulo-endothelial tissue cells.

Alteration in reproductive activity of the parasite—Acton and Chopra (1927) were the first to suggest that quinine acts by inhibiting the reproductive activity of the malarial parasites. Krishnan, Lal and Napier (1932) obtained further corroborative evidence in support of this view. In the course of the present studies the writer again noticed that after the administration of quinine whenever the cellular response was imperfect and there occurred a moderate increase of monocytes and intermediate forms without a corresponding increase in histiocytes there was evidence of interference with the reproductive activity of the parasite. Under the above-said circumstances the number of parasites in the peripheral blood invariably remained low and more or less constant. On investigation of the causes that lead to this low-grade infection it was found that there were two possible explanations (a) that the phagocytic mechanism being inadequate allows a small limited number of parasites reproduced at each cycle to gain an entry into red cells and complete their development, and (b) that the presence of the drug in the circulating blood retards the growth and development of a certain number of parasites. Repeated examinations of blood of these animals showed that early in the developmental cycle the ring forms persisted for a much longer time than usual and a great many of them showed no evidence of development even after 8 hours and that towards the close of the cycle a large number of dividing forms were continually present up to 32 hours after sporulation. These two observations suggest either that different broods of parasites maturing at different times were present or that the process of asexual maturation and division was being delayed in a certain percentage of the parasites. Although the writer believes that ordinarily the time taken for completing the asexual cycle is largely determined by the genetical make up of the parasite, he cannot, in view of the above observations, help presuming that inefficient action of quinine as

well as altered conditions in the host do prolong the time taken for asexual development by a few hours

In association with this delay in the cycle one often notices an increase in relative proportion of sexual forms. It is difficult to say whether the delay in development referred to is the cause of the reversion of some of the parasites to sexual forms or itself due to it. If sexual forms represent the end result of the parasite's successful reaction to an altered environment then imperfect action of quinine and inefficient immunity response may lead to the production of sexual forms. The writer has already pointed out that alterations in the reproductive activity of the parasite are generally associated with increase of monocytes and intermediate forms. Although the rôle of these cells in the production of an increase in the number of sexual forms is rather difficult to guess, it may be suggested that they bring about certain biochemical changes and these in turn lead to the parasite's reversal to sexual forms.

Acceleration of anti-body production—Some workers have suggested that anti-bodies play a part in overcoming malarial infection and that one of the ways in which quinine acts is by stimulating anti-body production. Although so far no one has been able to demonstrate definitely the presence of protective anti-bodies in malaria, there is no reason to suppose that anti-bodies are not formed. With each febrile attack a certain amount of foreign protein (malarial antigen) is liberated into the circulating blood, and anti-bodies to this will naturally be produced in varying amounts. Such a surmise is not mere theory only but is supported by a number of laboratory findings. Serological investigations have demonstrated the presence of anti-bodies through complement-fixation, precipitin and dermal (Sinton and Mulligan, 1932) tests. Furthermore, in the course of the present studies the writer has gained the impression that humoral changes in the serum are associated with increase of monocytes and intermediate forms, and that these cells are in some way connected with anti-body production. What one is not sure about is the function of anti-body in malaria. Theoretically and from analogy at any rate it can be said that if and when specific anti-body is present it will be greatly helpful in enhancing the susceptibility of *free* (extra-cellular) malarial parasites (though not of those within red cells) to phagocytosis and destruction. From a practical point of view the writer has obtained evidence that suggests that anti-body in malaria is of the nature of a sensitizing agent and that one of its actions is to alter the reticulo-endothelial system cells, in such a way that they will respond to the introduction of the infecting agent (antigen) at a later date more rapidly and intensely than previously. In an earlier paper (*vide* Krishnan, Lal and Napier, 1932) the writer showed that natural resistance to malarial infection is associated with a rapid and marked cellular response chiefly of monocytes and intermediate forms. Again, similar sensitiveness of the reticulo-endothelial cells to reinfection is noticeable after cure of the primary infection in a great number of monkeys, it is probable that sessile anti-bodies play a part in it because of its high specificity. If it is so, then the part played by quinine in this sensitization is a negligible one. All it can be given credit for is that the drug, by stimulating proliferation and functional activation of monocytes and leading indirectly to an increased production of anti-bodies, helps in the sensitization of the cells of the reticulo-endothelial system.

Increased output of young erythrocytes into the circulation—In the course of examination of supra-vital preparations of malarial blood the writer noticed that

young red cells may be a factor in infection and immunity in malaria and that quinine by helping in the destruction of effete and infected red cells provides the stimulus for the production of young cells and when these cells are present in large numbers an unfavourable environment for the entry and development of malarial parasites within red cells is created

DISCUSSION

From the evidence presented in the foregoing pages it will be seen that the mechanism of cure and immunity in malaria is almost purely of a cellular nature. Every factor concerned in cure of the disease has been shown to be in some direct or indirect way connected with the functional efficiency of the large mononuclear cells of the reticulo-endothelial system. The two types of cells, monocytes and histiocytes, comprising this system are mainly responsible not only for causing the death and disintegration of malarial parasites by phagocytosis but also for producing humoral and other changes that are helpful in overcoming the malarial infection. The action of quinine in malaria is merely to accelerate the natural processes at work. These processes start working as soon as the infecting agent establishes itself in the host, and so long as they function adequately and effectively the infection is kept down and cure established. But when they fail or are defective the infection either progresses rapidly and terminates in death or persists as a low-grade relapsing type. The usefulness of quinine is chiefly dependent upon the degree to which the natural processes of immunity can be stimulated by the drug and not upon the quantity of the drug or its direct lethal action on malarial parasites. This conception of the mechanism of cure of malaria by quinine appears to explain convincingly practically every known phenomenon connected with treatment of the disease. It is hoped that the evidence presented in the foregoing pages will be found clear, convincing and acceptable.

CONCLUSIONS

From supra-vital studies of blood of 46 monkeys infected with *P. mur* (?) certain observations having an important bearing on the mechanism of cure in malaria have been made. On the basis of these the mode of action of quinine seems to be as follows —

- (1) By accelerating the natural immune processes of mobilization, proliferation and functional activation of the phagocytic large mononuclear cells composing the reticulo-endothelial system the drug causes rapid engulfment and effective destruction of the malarial parasites.
- (2) By bringing about an alteration in the electrical condition of parasites and infected red cells by direct action it increases the susceptibility of these to phagocytosis.
- (3) By slowing down asexual reproduction and occasionally leading to the formation of sexual forms, it checks the intensity of infection.
- (4) By indirectly leading to the production of humoral changes (anti-bodies) and to the sensitization of the cells of the reticulo-endothelial system it increases the resistance to reinfection.

- (5) By causing the removal of effete and old red cells and increasing the output of young red cells it renders the successful entry of parasites into these cells more difficult

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APPENDIX

TABLE II

Showing the mobilization of large mononuclear cells in the peripheral blood of monkeys suffering from malaria after the administration of quinine

Group	Serial number	Total large mononuclear cells per c mm before quinine administration	Total large mononuclear cells per c mm after quinine administration	Increase in percentage	RESULT
Reaction of <i>M. rhesus</i> monkeys to quinine	1	1,260	1,530	21.42	Recovery
	2	1,350	2,340	73.33	
	3	1,050	2,200	109.52	
	4	960	1,582	64.79	
	5	1,744	2,850	63.41	
	6	1,406	2,400	70.69	

TABLE II—*contd*

Group	Serial number	Total large mono nuclear cells per c mm before quinine administration	Total large mono nuclear cells per c mm after quinine administration	Increase in percentage	Result
Reaction of <i>M. rhesus</i> monkeys to quinine	7	1,400	2,850	103.57	Recovery
	8	1,350	2,400	77.77	
	9	1,260	1,700	34.92	
	10	920	1,400	52.17	
	11	960	1,400	45.83	
	12	650	1,520	133.84	
	13	1,350	2,112	56.44	
	14	990	1,800	81.81	
	15	1,700	2,800	64.71	
	16	1,138	2,210	94.20	
	17	2,160	3,600	66.66	
	18	2,625	3,360	28.00	
	19	1,440	2,400	66.66	
	20	1,680	3,080	83.33	
	21	1,870	2,350	25.66	
	22	2,240	5,580	149.10	
	23	1,050	2,550	142.85	
	24	2,600	4,400	69.23	
	25	2,400	3,300	37.50	
	26	600	2,000	233.33	
	27	560	1,375	145.53	
	28	900	1,550	72.22	
	29	600	1,600	166.66	
	30	900	1,280	42.22	
	31	1,300	2,520	93.84	
	32	1,325	2,406	81.58	
	33	960	1,530	59.37	

TABLE II—contd

Group	Serial number	Total large mono nuclear cells per c mm before quinine administration	Total large mono nuclear cells per c mm after quinine administration	Increase in percentage	RESULT
Reaction of <i>M. rhesus</i> monkeys to quinine	34	900	1 170	30 00	Recovery
	35	1 760	2 160	22 72	
	36	1 875	3,240	72 80	
	37	1 350	2 275	68 51	
	38	2 210	3 060	38 46	
	39	1 510	2 340	54 04	
	40	1 400	1 800	28 57	
	41	1,787	3,200	79 07	
	42	2,800	3 600	28 57	
	43	2 025	3 120	54 07	
	44	920	1 350	46 73	
Reaction of <i>M. rhesus</i> monkeys during natural immunity	1	800	3 600	350 00	Recovery
	2	900	4 200	366 66	
	3	960	2 625	173 43	
	4	1,280	3 120	143 75	
	5	600	2,800	366 66	
Reaction of <i>M. cyno molgus</i> monkeys to quinine	1	700	2,850	307 14	Recovery
	2	4 000	5 600	40 00	
	3	910	2 850	213 18	
	4	1 406	4 000	184 49	
	5	1 400	5 400	285 71	
	6	990	3 200	223 23	
	7	1,125	3 120	177 33	
	8	560	1 700	203 57	
	9	2,500	3 392	35 68	
	10	1,620	2,850	75 92	

TABLE II—*concl'd*

Group	Serial number	Total large mono nuclear cells per c mm before quinine administration	Total large mono nuclear cells per c mm after quinine administration	Increase in percentage	RESULT
Reaction of <i>M. cynomolgus</i> monkeys to quinine	11	1,808	3,740	106.85	Recovery
	12	2,250	2,800	24.44	
	13	1,440	2,210	53.47	
	14	2,160	5,400	150.00	
	15	2,310	4,650	101.29	
	16	1,350	5,400	300.00	
	17	990	2,850	187.87	
	18	2,800	3,740	33.57	
Reaction of <i>M. cynomolgus</i> monkeys during natural immunity	1	910	2,850	214.28	Recovery
	2	875	2,880	229.14	
	3	1,400	4,320	208.57	
Reaction of <i>M. rhesus</i> monkeys to quinine	1	780	900	+15.38	No improvement—death
	2	438	520	+18.72	
	3	600	400	-33.34	
	4	720	420	-41.67	
	5	600	445	-25.84	
	6	600	600	0.0	
	7	575	160	-72.18	
	8	1,075	720	-33.03	
	9	960	900	-6.25	
	10	600	650	+8.33	
	11	400	225	-43.75	
	12	3,570	1,800	-49.58	

CONTRIBUTIONS TO PROTOZOAL IMMUNITY

Part I

THE EFFECT OF SPLENECTOMY ON THE COURSE OF MALARIAL INFECTION IN MONKEYS

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INTRODUCTION

THE increasing recognition that an accurate knowledge of the mechanism of infection and immunity in kala-azar is a pre-requisite to a satisfactory solution of the transmission problem of that disease, led us to undertake an investigation on the nature of immunity in protozoal diseases. Although our studies are chiefly confined to two diseases, namely kala-azar and malaria, we have no doubt that the results obtained will have a far wider application than at present appreciated. We included malaria in our study, not only for the purpose of elucidating the broader principles involved in protozoal immunity and for getting a clearer and truer picture of kala-azar immunity in which our chief interest lies, but also for determining the conditions under which malaria predisposes to kala-azar. Some of our studies on the subject have just been completed and we are presenting the results in this series of articles.

HISTORICAL

Although splenectomy has been performed on men and monkeys infected with malaria the results obtained do not warrant any definite conclusions with regard to the effect of the operation on the course of infection. While some claim definite improvement after removal of enlarged spleens in cases of human malaria (Rosati, 1912, Cumston, 1919, Raison, 1920,—*vide* Tahaferro, 1929), others

maintain that it interferes with the defence mechanism of the body and leads to relapse and intensification of infection (Degorce, 1913, Strime, 1914, Sidgwick, 1925,—*vide* Taliaferro, 1929) In *M. rhesus* monkeys infected with *P. lochi*, Gonder and Rodenwaldt (1910) noted that splenectomy was followed by relapse and severe infection and later their finding was confirmed by Noguchi (1928) who reported relapse of malaria following splenectomy in one *rhesus* monkey Blanchard and Langeron (1911) and Leger and Boulliez (1913) using *P. vivax* obtained somewhat similar results From the available evidence on account of the smallness of the number of animals experimented upon, one is not in a position to assess correctly the rôle of the spleen or of the reticulo-endothelial system in malaria We therefore thought it would be worth while undertaking, on a large scale, an investigation on the effect of splenectomy on the course of malarial infection in monkeys of different species A perusal of the results recorded in this paper will show that our work has not only yielded very suggestive and almost conclusive results as regards the importance of the spleen and the reticulo-endothelial system in malaria but has also given answers to a few of the debated questions on relapse, re-infection and cure of malaria

MATERIAL AND TECHNIQUE

After the completion of the cytological, parasitological and pharmacological studies undertaken in connection with monkey malaria at the School of Tropical Medicine, Calcutta, a large number of animals that had previously suffered from malaria and for which there was no further use was available These animals were apparently in normal health and instead of killing them, as is customary, it was decided to utilize them in the proposed splenectomy experiment To make the study complete some monkeys that had not previously suffered from malaria were also collected from the same source In all 30 animals consisting of 14 *M. rhesus*, 14 *M. mus* and 2 *M. radiatus*, were available and of these 9 *rhesus*, 9 *mus* and 2 *radiatus*, had suffered from malaria previously and 5 *rhesus* and 5 *mus* had not Normal *radiatus* monkeys were not available and therefore could not be included in the experiment

The operation of splenectomy was performed as follows The animal was etherized, the abdomen shaved and painted with iodine, a vertical incision two inches long in the region of the spleen made, the organ pulled out through the opening with two fingers, the pancreas separated from the pedicle and the latter clamped, the important vessels tied (preferably separately with silk), the organ excised and the abdominal incision closed, using silk for suture The usual aseptic precautions were taken and there was only one death in the whole series as a result of the operation and this was due to hæmorrhage following slipping of the ligature of the splenic vessels This animal was replaced by another to make up the total number to thirty

Commencing from the day after splenectomy, the peripheral blood of those monkeys that had previously suffered from malaria was examined daily for a period of 10 to 15 days with a view to finding out whether any relapse of malaria occurred in them Those that showed no relapse along with the normal splenectomized were given 0.5 c.c. of diluted blood from an infected *mus* monkey Throughout the experiment the infective material for inoculation was obtained from an *mus* monkey that showed a fairly persistent and uniform degree of infection, the parasite count

in this was about 5 to 6 per microscopic field, or approximately 50,000 per c mm of blood. The dose of blood given to infect a monkey was calculated to contain about 10 million parasites.

The strain of plasmodium used in these experiments was the one that was discovered by Napier and Campbell (1932) in a *M. irus* monkey and believed to be almost identical with *P. vivax* (vide Sinton and Mulligan, 1932). This strain produces a severe and fatal infection in *M. rhesus* and a low grade infection with a tendency for spontaneous cure in *M. irus* and *M. radiatus*. This natural difference in the intensity of infection in the three species of monkeys has greatly helped us in this investigation and has clarified many a point which would otherwise have been still debatable.

As one of our chief objects was to determine correctly the effect of splenectomy on the course of malarial infection, we had to fix for purposes of comparison, a standard which would give a true picture of infection in non-splenectomized animals. For this we have utilized our observations on 45 infected monkeys—some of these animals have been used in the splenectomy experiments. We have shown the salient features of the infection in the different species side by side with those of splenectomized monkeys in Tables I, II and III. Our picture of infection in the non-splenectomized group completely corresponds with those obtained by other workers (vide Knowles and Das Gupta, 1932).

In order to obtain a correct idea regarding the effect of specific treatment on the course of infection in splenectomized and non-splenectomized monkeys, we standardized our method of treatment as follows. The drug used for treatment was hospital quinine and was obtained from the dispensary of the School of Tropical Medicine. Treatment was begun in the splenectomized group as soon as the parasite-rate reached about 500,000 per c mm of blood. If the animals failed to show this number they were left untreated to find out if spontaneous recovery would occur. The routine consisted in giving 1 grain of quinine in solution by intramuscular injection every morning and a powder consisting of 2 grains of quinine and 20 grains of soda bicarb by mouth every evening for one week. If the animal survived this period and showed signs of recovery the injection was stopped and two powders instead of one was administered by mouth for another week until disappearance of all parasites from the peripheral blood. After this no regular administration of quinine took place unless the parasite-rate went above 100,000 per c mm of blood and when this happened quinine powders were administered by mouth as previously.

RESULTS

Of the 30 monkeys splenectomized only 12 showed relapse of infection after the operation (detailed histories of these are furnished in Part II of the series), the remaining 18 had therefore to be given an inoculation of infective material to produce malaria in them. The course of infection in all the thirty monkeys, as well as the response to treatment of some of them were studied and the results are summarized in Tables I, II and III. In Tables I and II it will be seen that the splenectomized monkeys are further subdivided into two groups (1) those with a history of previous malaria and (2) those with no history of previous malaria. This division was found necessary because the course of infection was much more severe and the response to treatment distinctly poorer in the latter than in the former group.

TABLE I

Showing the course of malarial infection in non-splenectomized and splenectomized *M. rhesus*

	Non splenectomized group	Splenectomized group with a history of previous malaria	Splenectomized group with no history of previous malaria
Total number of monkeys studied	27	9	5
Average height of parasite count per c mm of blood.	Over 1 million	Over 1 million	Over 2 millions
Percentage of infected red cells	Over 80 per cent	Over 90 per cent	Over 90 per cent
Average number of days between date of inoculation and date of highest count	10 7 ± 0 2890	8 8 ± 0 4305	7 2 ± 0 3529
Description of infection and illness	Acute severe illness Rapid development Heavy parasitization More than 1 parasite per corpuscle common Highly fatal	Acute severe illness Rapid development Heavy parasitization More than 2 parasites per corpuscle very common Highly fatal	Acute severe illness Very rapid development Very heavy parasitization 3 to 6 parasites per corpuscle very common Highly fatal
Termination in untreated	Number of animals studied 7 Death rate 71.4 per cent Showing few parasites in blood 28.6 Showing no parasites in blood 0 Hemoglobinuria 28.6 Number of animals studied 20 Death rate 20 per cent Showing few parasites in blood 20 Showing no parasites in blood 60 Hemoglobinuria 15	Number of animals studied 3 Death rate 100 per cent Showing few parasites in blood 0 Showing no parasites in blood 0 Hemoglobinuria 100 Number of animals studied 6 Death rate 83.3 per cent Showing few parasites in blood 16.7 Showing no parasites in blood 0 Hemoglobinuria 50	Number of animals studied 2 Death rate 100 per cent Showing few parasites in blood 0 Showing no parasites in blood 0 Hemoglobinuria 100 Number of animals studied 3 Death rate 100 per cent Showing few parasites in blood 0 Showing no parasites in blood 0 Hemoglobinuria 66.7
Termination in treated			

TABLE II

Showing the course of malarial infection in non-splenectomized and splenectomized Monkeys

	Non splenectomized monkeys	Splenectomized monkeys with a history of previous malaria	Splenectomized monkeys with no history of previous malaria
Total number of monkeys studied	14	9	5
Average height of parasite count per c mm of blood	Below 50,000	0.5 to 2 millions	0.5 to 2 millions
Percentage of infected red cells	Below 2 per cent	30 to 50 per cent	Over 70 per cent
Average number of days between date of inoculation and date of highest count	23.3 ± 1.2310	8 ± 0.0119	6.6 ± 0.1444
Description of infection and illness	Mild or no illness Slow development Low parasitization More than 1 parasite per corpuscle uncommon Not at all fatal	Acute severe illness Rapid development Heavy parasitization More than 1 parasite per corpuscle common Highly fatal if untreated	Acute severe illness Very rapid development Very heavy parasitization 2 to 4 parasites per corpuscle very common Highly fatal
Termination in untreated	Number of animals studied 10 Death rate 0 per cent Showing few parasites in blood 60 " Showing no parasites in blood 40 " Haemoglobinuria 0 " Number of animals studied 4 Death rate 0 per cent Showing few parasites in blood 0 " Showing no parasites in blood 100 " Haemoglobinuria 0 "	Number of animals studied 7 Death rate 57.1 per cent Showing few parasites in blood 42.9 " Showing no parasites in blood 0 " Haemoglobinuria 42.9 " Number of animals studied 2 Death rate 50 per cent Showing few parasites in blood 0 " Showing no parasites in blood 50 " Haemoglobinuria 50 "	Number of animals studied 2 Death rate 100 per cent Showing few parasites in blood 0 " Showing no parasites in blood 0 " Haemoglobinuria 100 " Number of animals studied 3 Death rate 33.3 per cent Showing few parasites in blood 33.3 " Showing no parasites in blood 33.3 " Haemoglobinuria 0 "
Termination in treated			

TABLE III

Showing the course of malarial infection in non-splenectomized and splenectomized M. radiatus

	Non splenectomized group		Splenectomized group with a history of previous malaria	
Total number of monkeys studied	4		2	
Average height of parasite count per c mm of blood	Below 20,000		Over 1 million	
Percentage of infected red cells	Below 0.75 per cent		50 to 70 per cent	
Average number of days between date of inoculation and date of highest count	21±0.6306		8.5±0.2384	
Description of infection and illness	Very mild or no illness Very slow development Low parasitization More than 1 parasite per corpuscle very uncommon Not at all fatal		Acute severe illness Rapid development Heavy parasitization More than 2 and 3 parasites per corpuscle quite common Highly fatal if untreated	
Termination in untreated	Number of animals studied 2 Death rate 0 per cent Showing few parasites in blood 0 " Showing no parasites in blood 100 " Hæmoglobinuria 0 "		Number of animals studied 1 Death rate 100 per cent Showing few parasites in blood 0 " Showing no parasites in blood 0 " Hæmoglobinuria 100 "	
Termination in treated	Number of animals studied 2 Death rate 0 per cent Showing few parasites in blood 0 " Showing no parasites in blood 100 " Hæmoglobinuria 0 "		Number of animals studied 1 Death rate 0 per cent Showing few parasites in blood 0 " Showing no parasites in blood 100 " Hæmoglobinuria 0 "	

From Table I it will be seen that as regards (1) 'the height of parasite count reached', and (2) 'the percentage of infected red cells' there is no real difference between the splenectomized and non-splenectomized groups. But a careful comparison of the rest of the table shows that in the splenectomized group (i) the number of days taken to reach the highest parasite count is distinctly less by over 25 per cent, (ii) the number of parasites per red blood corpuscle is very much greater and as many as 4 to 6 parasites in one red cell were quite frequently met with, (iii) the death rate both in the treated and in the untreated is significantly higher, being

83 and 100 per cent as against 20 and 71 per cent respectively, (iv) the response to treatment is very poor and in no case has it been possible to rid the peripheral blood completely of all parasites, (v) and finally the incidence of hæmoglobinuria is extremely high, being 50 to 100 per cent as against 15 to 28 per cent in the non-splenectomized group

It will be remembered that the *rhesus* monkey, normally, is very susceptible to malaria and suffers from a severe and fatal infection even when the spleen is present. From the point of view of splenectomy experiment therefore it is not the most suitable animal, and the differences enumerated above are all that could be expected. They should be considered as quite suggestive because these results assume far greater significance when considered along with the results obtained from the other two species.

As the results obtained from *irus* and *radiatus* monkeys are almost identical, Tables II and III may be taken up for consideration together. A glance at these two tables will show the very pronounced and striking effect of splenectomy on the course of malarial infection in these two species. While a low grade infection with 20 to 50 thousand parasites per c mm of blood, and 1 to 2 per cent of infected red cells, is the chief feature of infection in the normal, a severe acute infection with one million parasites per c mm and 50 to 70 per cent infected red cells is the characteristic type in the splenectomized animals. The time taken for reaching the highest parasite count is reduced from three weeks in the non-splenectomized to one week in the splenectomized monkeys. This is evidence to show that the number of surviving parasites after each schizogony cycle is very much greater in splenectomized than in non-splenectomized animals, this is probably due to a failure of the phagocytic immunity mechanism in the former. Again, multiple infection of red blood corpuscles which is rare in the non-splenectomized is a feature in the splenectomized animals. Death and hæmoglobinuria due to malarial infection are unknown in normals, while they range from 50 to 100 per cent in the splenectomized animals. Favourable response to treatment that is obtainable in 100 per cent of normal animals is not seen even in 50 per cent of splenectomized animals.

It will be seen from the above description that the picture of infection in the *irus* and *radiatus* monkeys after splenectomy is identical with that of the *rhesus* monkey. The marked differences in the intensity of infection due to differences in species noticeable in normals are completely absent in the splenectomized. This observation furnishes proof that the spleen and the reticulo-endothelial system are of very great importance in malarial immunity, both natural and acquired.

DISCUSSION

In spite of the large amount of experimental evidence produced by different workers (Cannon and Taliaferro 1931, Napier, Krishnan and Lal, 1932, Krishnan, Lal and Napier, 1933) there appears to be still some difference of opinion as regards the importance of the spleen and the reticulo-endothelial system in malarial immunity. At a recent meeting of the Royal Society of Tropical Medicine, in which this subject was discussed (vide *Brit Med Jour* March 1933, p 368), Professor Thomson and Dr Hamilton Farley stressed the importance of the reticulo-endothelial system in malaria while Professor Warrington Yorke

opined that he did not think the system played any essential part in the development of 'tolerance' in malaria but that its function was merely that of a 'scavenger' to clear up debris. As a result of cytological studies conducted with the help of the supra-vital staining technique, we (Krishnan, Lal and Napier, 1933, Krishnan, 1933) produced evidence that clearly indicated the very important part played by the two types of phagocytic large mononuclear cells, histiocytes and monocytes, composing the reticulo-endothelial system in overcoming malarial infection. In that we showed that the histiocytes were primarily responsible for phagocytosis and disposal of infected red cells and the monocytes for engulfment and destruction of free malarial parasites. To us it appears that the reason for the prevalence of the idea that the reticulo-endothelial system plays the part of a 'scavenger' is because of the behaviour of histiocytes. One of the accepted normal functions of these cells whether in the blood or in the tissues is the disposal of damaged and degenerated red blood corpuscles. This does not necessarily mean that their function in malaria is only 'scavenging'. No doubt it is part of their function but it is not the whole of it. We ourselves pointed out that the histiocytes probably take up infected red blood cells in malaria not for the purpose of destroying the parasites contained in them but rather for the disposal of the damaged red cells containing them, and that the death of the ingested parasites within these cells when it takes place may be purely accidental or incidental, due to the unsuitability of the conditions within these cells rather than to any inherent lytic powers possessed by them. These arguments, while lending a limited degree of support to the 'scavenger' theory of histiocytes, do not appear to us in any way to lessen the importance, either of these cells or of the reticulo-endothelial system in malarial immunity. For, after all, one has to take into consideration the ultimate result on the malarial parasites, of phagocytosis of infected red cells by histiocytes, and also the part played in overcoming malaria by cells other than the histiocytes that compose the reticulo-endothelial system.

The function of the histiocyte does not represent the whole function of the reticulo-endothelial system, our cytological studies have shown that the histiocytes, monocytes, and the intermediate forms all play important parts and jointly overcome the malarial infection. In this paper we have presented what we consider as strong evidence in favour of the above view. The removal of the spleen which is perhaps richest in reticulo-endothelial tissue, has been shown to result in a failure of natural and acquired resistance to malaria and to cause an increase, both in the severity of infection as well as in the mortality rate. To us the most suggestive evidence in this direction is that obtained from the studies of the *urus* and *radiatus* groups. In these animals splenectomy has been responsible for the conversion of a low grade infection that normally ends in spontaneous recovery, into one of extreme severity and of high mortality accompanied by hæmoglobinuria. The marked beneficial results of specific treatment noticeable in non-splenectomized monkeys are no more found in the splenectomized animals. This stresses the importance of the spleen and of the reticulo-endothelial system in malarial immunity.

It may be asked, if the spleen is so very important in resistance to malaria how does partial recovery take place after treatment in a certain number of splenectomized animals. The answer to this question is that although the spleen is the

richest in reticulo-endothelial tissue, it is not the only organ in which the tissue is present. Cells of the reticulo-endothelial system have a very wide distribution and are to be found in several parts of the body other than the spleen, e.g., liver, lymphatic gland, adrenals, pituitary, blood and connective tissue. Even after the removal of the spleen the reticulo-endothelial tissue outside the spleen will continue to proliferate and function. The results of splenectomy will therefore depend entirely upon (a) the amount and (b) the functional efficiency of the reticulo-endothelial tissue outside the spleen. These, in turn, will vary with the species of animals and its previous immunological history.

If this view is accepted then it is easy to understand why treatment of splenectomized monkeys of different species has yielded slightly different results. Cytological studies of the splenectomized animals (not recorded here) show that, on the administration of quinine there is a greater response of reticulo-endothelial cells in the *irus* and *radatus* than in the *rhesus* and that the response of those that gave a history of previous malaria was greater than those that gave no such history. This corroborates the validity of our view that previous experience in overcoming malaria evidently sensitizes the whole of the reticulo-endothelial system in a way such that it could respond to subsequent infection and treatment more readily than before and that it is this sensitization that is responsible for the partial recovery in some of the splenectomized animals.

Taking the results presented above as a whole there appears to be little doubt that one of the chief factors that controls the intensity of malarial infection and leads to recovery from that disease is the functional efficiency of the reticulo-endothelial system. Upon its powers of mobilization, proliferation and functional activation depend immunity in malaria. Removal of the spleen, by interfering with these processes, converts latency into relapse, resistance into susceptibility and recovery into death.

SUMMARY AND CONCLUSIONS

The course of malarial infection in 30 splenectomized and 45 non-splenectomized monkeys belonging to three different species was studied.

It was found that non-splenectomized monkeys developed a moderately severe or mild infection and responded readily to specific treatment, while splenectomized animals suffered from a severe, rapidly fatal infection accompanied by hæmoglobinuria and did not respond to specific treatment.

From this it is concluded that the spleen and the reticulo-endothelial system are of the greatest importance in malarial immunity, both natural and acquired.

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OBSERVATIONS ON CIRRHOSIS OF THE LIVER AS SEEN IN THE PUNJAB

BY

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IN a previous paper (Hughes and Shrivastava, 1927) some account was given of the cirrhosis of the liver which occurs in the Punjab, and the opinion was expressed that it was probably the result of a pathological process similar to that described by Banti in the disease which is called after him and that malaria played an important part in its causation. The condition is fairly common in the Punjab and in Northern India generally, as appears from the following figures taken from the records of the Mayo Hospital, Lahore, an institution which draws patients from all parts of the Province and to some extent from outside it —

Period	Total admissions to medical wards	Admissions on account of cirrhosis of the liver
1929-1932	7,911	241

Patients usually seek admission when portal obstruction is well established but opportunities sometimes occur of observing the earlier stages of the disease. Death probably takes place more frequently from intercurrent infections than from liver failure but it is not possible to give any figures, as patients do not as a rule stay long in hospital, and in any case are generally removed to their homes when their condition becomes bad. Post-mortem examinations are consequently difficult to obtain. Of 30 cases forming the main basis of this communication only three died in hospital and of these two came to autopsy.

The clinical picture of the fully established disease is in general that of portal or multi-lobular cirrhosis. The liver varies in size according to the degree of fibrous shrinkage. At first smooth, it eventually becomes nodular. The spleen is generally more or less enlarged and may be tender from the presence of perisplenitis. If palpable it has a hard, non-resistant feel. A history of

hæmatemesis is occasionally obtained but complications related to the collateral venous circulation are not common. Microscopically the liver shows the changes characteristic of multi-lobular cirrhosis and the spleen those produced by chronic malaria. In old standing cases the connective tissue in the liver contains few cells but in more recent cases this tissue may be very cellular. In these the portal spaces are widened and some proliferation or infiltration of cells is seen extending into the lobules. The process is, however, essentially a portal cirrhosis and not a cirrhosis of the 'intracellular' type such as occurs in kala-azar (Rogers, 1930). The changes in the spleen also vary with the duration of the disease. The organ may be almost completely composed of hard shrunken fibrous tissue and be little or not at all enlarged. On the other hand it may be much increased in size and have a 'beefy' appearance on section. Microscopically the pulp in these cases is seen to be replaced to a large extent by proliferated reticular and endothelial cells with a variable amount of fibrous tissue. In the more shrunken spleens the Malpighian bodies are fibrosed, the central vessels becoming eventually obliterated. The capsule and trabeculae are thickened and there is often thickening of the walls of the splenic veins (sclerosing endophlebitis). Blood-derived pigments are always present and malarial pigment is frequently seen. During the course of the disease attacks of malarial fever may occur but more often there are bouts of low irregular temperature not responding to quinine, the blood being negative for malarial parasites.

The present communication is concerned (1) with observations on certain biochemical and other features of the fully developed disease, (2) with variations in the clinical picture, and (3) with the evolution of the disease process and other points which bear on the question of ætiology.

Bilirubinæmia—A finding which is very frequent (23 out of 28 cases), and to which attention was drawn on previous occasions (Hughes, 1924, Hughes and Shrivastava, 1927), is the occurrence in the blood of excess of bile pigment giving the indirect van den Bergh reaction. In the present series of cases the amount varied from 1 to 4 van den Bergh units. Urobilinuria and normal or exaggerated pigmentation of the stools were always associated with this hyper-bilirubinæmia. These facts suggest increased destruction of red blood cells by the hyperplastic reticulo-endothelial cells of the spleen, although it must be remembered that the urobilinuria may have been due to some extent to insufficiency of the liver and to the escape of portal blood into the general circulation through anastomotic channels (Rolleston and McNee, 1929). Defective hepatic function may also have been partly accountable for the increase of bile pigment in the blood. In cases without any hyper-bilirubinæmia the spleen was either not palpable at all or only slightly enlarged and very hard, an indication that the erythrocyte-destroying activities of that organ were probably abolished by overgrowth of fibrous tissue. Manifest jaundice was present in some patients as a temporary feature, evidently due to damage to the liver cells, the pigment in the blood being of the 'biphasic' variety.

Blood proteins—Estimations of the blood proteins were carried out on 8 patients, 3 of whom had œdema of the legs. Six were free from albuminuria and two showed faint traces. The albumin/globulin ratio was below normal in all. It varied from 1.4 to 0.53. The serum albumin was subnormal in 7 (3.07 to 2.2 per cent) but there was no reduction in the total proteins in any patient (7.28 to 6.0 per cent). The amount of fibrin was normal in every case except one in whom it

was only 0.05 per cent. The methods employed were those of Hawk and Bergheim (1926). Oedema with lowering of the blood protein content and of the albumin/globulin ratio has been described by Bennett, Dodds and Robertson (1930) in cases of portal cirrhosis without evidence of kidney damage. In view of the relationship between the level of the blood proteins, especially of the serum albumin and the occurrence of dropsy (Epstein, 1917, 1922) there can be little doubt that the oedema of the legs so frequently associated with cirrhosis of the liver is at least as much the result of hypoproteinaemia as of pressure on the intra-abdominal veins, the explanation usually offered.

Contractility of the spleen—Observations on the contractility of the spleen were made in 16 cases by noting the changes in the red and white cell counts after injecting 1 c.c. of adrenalin (1 in 1,000) subcutaneously. The method used was that previously employed in studying the enlarged malarial spleen (Hughes and Shrivastava, 1930). Some typical results are given in the following table—

Case	RED CELLS PER CMM (MILLIONS)		WHITE CELLS PER CMM	
	Before adrenalin	20 minutes after adrenalin	Before adrenalin	20 minutes after adrenalin
1	7.60	7.74	12,600	12,560
2	6.36	6.97	8,430	10,800
3	5.57	5.88	11,330	13,900
4	3.06	3.34	5,670	8,700
5	5.24	5.76	7,070	16,130
6	3.40	3.66	5,700	12,360
7	5.43	5.47	4,530	10,900
8	5.69	5.99	5,800	8,100
N o r m a l s				
1	5.99	7.30	7,930	10,830
2	5.75	7.05	7,770	13,670
Normal recently splenectomized				
1	5.50	5.61	8,100	8,330

In most patients the rise in red cells was much less than normal. This was so even when the spleen was not enlarged. In fact the rise was least marked in such cases, an indication that the relatively small size of the spleen was due to contraction of fibrous tissue which almost completely abolished the function of the organ as a hæmoglobin reservoir. This indirect evidence of the condition of the spleen is confirmed by post-mortem findings. The changes in the total leucocyte count

did not always run parallel to those in the numbers of erythrocytes. In certain advanced cases a large increase, up to 100 per cent or more, in white cells accompanied a small rise in red cells, the chief alteration in the differential count being a small rise in the proportion of lymphocytes at the expense of the polymorphonuclears. The extra leucocytes could not have come from the spleen in these patients. The lymph glands suggest themselves as likely sources. In some individuals the total red cells were much reduced in number while in others a condition of apparent erythæmia existed. This was usually seen in advanced cases and was probably due to concentration of the blood. The occurrence in such patients of increased resistance of the red cells to saline hæmolysis was confirmed (Hughes and Shrivastava, 1927).

Ascitic fluid—The specific gravity and the protein and cell content of the ascitic fluid were observed in 14 cases. Protein was estimated by Esbach's method and was found to vary from 0.03 to 1 per cent, figures lower than those given by Rolleston and McNee (*loc cit*) for the ascitic fluid in portal cirrhosis. The specific gravity ranged from 1.010 to 1.020. The cell content was variable. In some cases epithelial cells predominated and in others leucocytes (mostly lymphocytes). Red blood cells in small number were seen in all fluids except one. Rolleston and McNee (*loc cit*) suggest that the presence of red cells in these fluids is the result of injury to vascular adhesions during previous tapping, or rarely of peritonitis associated with alcoholism or of acute infections. Erythrocytes were, however, found in cases that had not been previously tapped and that had no signs of peritonitis or of acute infection.

Renal efficiency—The urea clearance test for renal efficiency was done on 14 cases by the method of Austin, Stillman and van Slyke (1921). This examination was suggested by the presence of albuminuria in some patients, by the fact that previously (Hughes and Malik, 1932) portal cirrhosis had been found at autopsy in a patient who died from renal failure resulting from chronic hæmorrhagic nephritis, and by the presence of patchy fibrosis of the kidneys in two other cases. The 'standard' clearance was determined and the findings indicated definite defect in kidney function in 6 cases, viz., clearances of under 30 c.c., the average normal for adults being 54 c.c. Proteinuria varying from a trace to 0.6 per cent was present in four patients. It is possible that in some advanced cases concentration of the blood may to a certain extent be responsible for the lowered kidney efficiency.

Glycogenic function—In the series of cases now being reported no tests of the glycogenic function of the liver were carried out. Previous experience (Hughes and Malik, 1927), however, showed that defective storage of carbohydrates is common not only in our hospital patients who have cirrhosis of the liver but also in those who have not. The significance of this finding has already been discussed (Hughes and Malik, *loc cit*).

Clinical features—In the fully developed cases the history, symptoms, and physical signs usually render the diagnosis obvious. Sometimes, however, atypical cases are seen. The association in one case of portal cirrhosis with advanced hæmorrhagic nephritis has been mentioned as well as the occurrence of œdema, with or without proteinuria, in many instances. In these patients the disappearance or diminution of the extra-abdominal dropsy usually unmasks the ascites resulting from portal obstruction and in some of them an enlarged liver may be detected, often with an enlarged hard spleen. Ascites, due to tubercular or other forms of

chronic peritonitis or to congestive heart failure, seldom gives rise to trouble in diagnosis but heart failure is sometimes the terminal event in old standing cirrhosis patients

In most cases the abdominal swelling is said to have developed in one to six or seven months. Occasionally there is a previous history of ascites which disappeared perhaps after tapping, and was followed by a long latent interval during which there was no accumulation of fluid in the abdomen. In one patient this interval was as long as four years. In such a case one may assume that the temporary disappearance of ascites was due to the development of the collateral circulation which kept pace for a time with the obstruction to the portal flow but was eventually insufficient for this purpose.

Hæmatemesis sometimes occurs in patients in whom there is little or no abdominal fluid and in these the diagnosis may be difficult until ascites begins to develop. Thus —

A R., aged 45, Muslim male who had suffered considerably from 'fever', was admitted to hospital on 7th March 1933 with a history of having had repeated attacks of hæmatemesis and melena during the previous ten days. He was very weak and anæmic, the red cells being only 2½ millions per cmm. and the hæmoglobin 50 per cent. The leucocytes numbered just over 4 000 per cmm. The conjunctivæ were slightly icteric. The urine contained much urobilin and the blood serum gave a strong indirect van den Bergh reaction. The stools were deeply coloured and contained occult blood. No enlargement of the liver could be made out but the spleen was 4 fingers below the costal margin. It was hard and tender (perisplenitis) with an irregular surface. The abdomen was lax and the superficial veins not prominent. The peritoneal cavity contained a little fluid. The history, symptoms and physical signs (including X ray examination) did not suggest peptic ulceration. The W R was negative and no amebic cysts or ova were found in the stools. The patient ran a temperature of 99.1–100.0° for five days after admission. Malarial parasites were not found in the blood but quinine (30 grains daily) and plasmoquine (½ grain daily) were given for a period of 3 weeks. Sixty grains a day of ferri et ammonii cit were given during the whole of the patient's stay in hospital. He left on 27th April, 1933. From 17th March 1933 there was no temperature nor was there any sign of recurrence of the bleeding. The ascitic fluid increased in amount up to about 19th March, 1933, when a considerable quantity was present. Thereafter it decreased somewhat but never disappeared. Tapping was not necessary. The abdominal veins were more prominent on discharge, than on admission. There was no evidence of hæmorrhoids. The patient's general condition on discharge was fairly good.

In some patients the amount of abdominal fluid remains stationary even when contraction of the liver and spleen and increasing prominence of the abdominal veins indicates progressive portal obstruction. Here again there is obviously a balance between the collateral drainage and the degree of obstruction.

Ætiology —The grounds on which it has been suggested that malaria plays a prominent part in the causation of this condition are the following: the histories of patients show that they all have suffered from repeated attacks of malaria, mostly seasonal in incidence and treated insufficiently or not at all, malaria is known to damage the liver cells (Sinton and Hughes, 1924, Williams, 1927), enlargement of the spleen often precedes clinically detectable changes in the liver, post-mortem examination has revealed chronic malarial changes in the spleen in every case of mine so far examined (about 15 in all), in patients in whom no splenic enlargement can be detected during life, there is indirect evidence of fibrosis of the spleen from the effects of adrenalin on the blood count, the pathological changes in the portal spaces of the liver are similar to those in the pulp of the spleen, viz, cellular hyperplasia with more or

less fibrosis. It can of course be argued that the presence of an enlarged malarial spleen is no proof of the malarial origin of this condition, as in the population to which these patients belong malaria is practically universal and consequently enlarged malarial spleens are very common. Such spleens are, however, not as common among the general population, either inside or outside hospital, as they are among those whose livers are cirrhotic. Also, in the cases that go on to cirrhosis enlargement of the liver usually accompanies the splenomegaly. Rogers (*loc cit*) has suggested, as a result of post-mortem observations, that cirrhosis of the liver in Bengal is due to amœbiasis. In the Punjab, however, I have not found that amœbic infection is any commoner among patients with cirrhosis of the liver than it is among the general hospital population. Repeated examination of the stools of 30 cirrhosis patients showed amœbæ in only 2. Syphilis would not seem to be a causative factor either. In the vast majority of cases there is no history of syphilis, the post-mortem findings are not typical of syphilis and anti-syphilitic treatment has no effect on the progress of the disease. Among 30 cases the W R was positive in two only. Alcohol can also be ruled out as an ætiological agent as most of the patients never take alcohol in any form. So also can hookworm infection, which was absent from most of the cases examined*. On the other hand spiced and irritating foods may play a causative rôle and the presence in some patients of fever of obscure origin, untouched by quinine, suggests that bacterial infection may also assist in the production of the disease. The occurrence of jaundice of a toxic nature in certain cases with no evidence of active malaria points to the intermittent action of some non-malarial poison on the liver cells. This fact also supports the conclusion of Rolleston and McNee (1929) and of McNee (1932) that in portal cirrhosis the causative agent acts both on the parenchymatous and on the supporting tissues of the liver. The lesions produced are, like those resulting from X-rays (Bolliger and Inglis, 1933), of a type from which functionally adequate regeneration of the liver-cells is not possible. The severe toxic jaundice sometimes seen in the Punjab at the end of a bad malarial autumn (Hughes, 1926) may be related to cirrhosis of the liver.

It may be objected that malarial parasites are not generally found in these patients during life or after death. It is not contended, however, that continual active malarial infection is necessary for the progress of the disease. The enlarged spleen of chronic malaria is due to the proliferative response to malarial infection of the reticulo-endothelial cells and fibroblasts of that organ and it generally persists in some degree after the infection is destroyed. In certain cases the hyperplastic and overactive splenic cells continue to destroy red blood corpuscles and a form of hæmolytic splenic anæmia results (Hughes and Shrivastava, 1931), while in others fibrosis and shrinkage take place. Changes similar to the latter are responsible for the replacement of the highly cellular tissue occupying the portal spaces in early cases of cirrhosis by the fully formed and almost non-cellular connective tissue seen in old standing cases. The fibrosis, having been thus started, may be continued by other toxic agents such as those mentioned above, after the malaria has been eradicated.

Banti described three stages of the disease which is called after him, viz, (1) chronic enlargement of the spleen with anæmia, (2) enlargement of both spleen and

* Schistosomiasis, a cause of cirrhosis of the liver in Egypt, does not exist in the Punjab

liver with subicterus and urobilinuria, the liver being at first smooth, and (3) cirrhosis of the liver with ascites and perhaps jaundice. The same three stages, or at least the last two of them, exist in the condition under discussion and hence it might be classified as a form of Banti's syndrome. The following case illustrates the gradual change from the second to the final stage or from 'chronic malaria with enlargement of the spleen and liver' to 'cirrhosis of the liver with ascites'—

Z, aged 12, Muslim female, who is still (6th June 1933) in hospital, was admitted on 14th March, 1933, on account of general weakness and with marked swelling of the abdomen. There was a history of irregular fever with rigors over a period of 3 or 4 years. No hæmatemesis or melena. The patient was anæmic with only 2½ millions red cells per c mm and 55 per cent hæmoglobin. The abdominal swelling was chiefly due to the presence of a very large, hard spleen which extended downwards into the left iliac fossa and across the middle line. The liver was also enlarged about two inches below the costal margin and had a resistant feel. Neither organ was tender. The urine contained a trace of albumin and much urobilin. The stools were deeply pigmented and contained a few hookworm ova. The blood serum gave a positive indirect van den Bergh reaction. The W R was negative. Hookworms were successfully dealt with by the administration of calcium tetrachloride and oil of chenopodium. Treatment otherwise has consisted chiefly in the giving of quinine, plasmoquine and iron. The patient has had no fever while in hospital.

During the past 2½ months the liver has become gradually smaller till at present (6th June, 1933) it is barely palpable under the costal arch. The spleen has shrunk also but comparatively little. Fluid has accumulated in considerable quantity in the abdomen. A sample of this fluid showed characters similar to those of the ascitic fluid in cirrhosis of the liver. The abdominal veins have become prominent and altogether the case is now typical of portal cirrhosis with much enlargement of the spleen. There are no hæmorrhoids and frank jaundice has never been seen.

One is, however, inclined to agree with the suggestion of McNee (*loc cit*) 'that the term "Banti's disease" should be deleted from our nomenclature' and that the conditions usually so designated should be 'classified simply as the late stage of splenic anæmia or what is perhaps more apt, as unusual types of ordinary portal cirrhosis in which high portal blood-pressure and changes in the spleen precede the onset of marked fibrotic changes in the liver'. Looked at from the latter point of view the cirrhosis of the liver found in Northern India differs from ordinary portal cirrhosis only in the fact that the initiating if not the chief, causative agent, viz., repeated attacks of malaria, is one which usually produces marked specific changes in the spleen before fibrosis occurs in the liver.

SUMMARY

A general account, mainly based on 30 cases, is given of the cirrhosis of the liver seen in Northern India and the cause of the condition is discussed, with special reference to the part played by malaria. It is concluded that repeated attacks of this disease constitute an important, if not the chief, ætiological factor. Investigations into the following features of the condition are described, the presence of hyperbilirubinæmia, diminished contractility of the spleen, diminution of the blood proteins and of the albumin/globulin ratio and lowered efficiency of the kidney as shown by the urea clearance test. Certain characters of the ascitic fluid are also described.

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AN INVESTIGATION ON THE DIAGNOSTIC VALUE OF SERUM PROTEIN CHANGES IN KALA-AZAR

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INTRODUCTION

FROM the days of Donovan onwards it has been the tradition of the Madras General Hospital as far as possible to avoid spleen puncture in arriving at a diagnosis of kala-azar. We are aware that in Calcutta spleen puncture is said to be done with safety even on out-patients but an occasional, nay a rare, fatality has in Madras acted as deterrent to resort to spleen puncture as a routine in clinching a diagnosis of kala-azar. Short of a patient, painstaking search for parasites in peripheral blood, which one does rarely, one has been relying as a routine measure on history, the temperature chart, the red and white blood corpuscular ratio, the formol-gel reaction, the response to treatment and so on.

There have been occasions when one felt the need for a more consistent and reliable diagnostic measure, short of finding the Leishman-Donovan bodies on spleen puncture. The gel reaction does not become positive for a period of five months and even afterwards one comes across cases in which it does not definitely help in diagnosis. The serum antimony test has been found fallacious here (Guruswami Mudaliyar, 1924). The gel reaction is generally considered to be due

to change in quality of the serum euglobulin associated with quantitative increase (Lloyd and Paul, 1928) Chopia and Choudhury (1929, 1931) argue that gelation and opacity formation—the two components in the leucogel reaction—may be due to two different proteins, of which opacity alone may perhaps be due to euglobulin or another substance associated with it and sharing its properties, and that hydrogen-ion concentration has been ignored by previous workers in assessing the mechanism of the gel reaction. They also suggest that gelation may be associated with destruction of red corpuscles and opacity protein may result from the destruction of white blood cells, which is so excessive in kala-azar and on which is based the diagnostic importance in the disease of the red and white blood corpuscular ratio.

INVESTIGATION DONE

The significance of serial protein estimations in controlling the response to treatment has been traced in kala-azar (Lloyd and Paul, 1928, Lloyd, Napier and Paul, 1929), in malaria and typhoid (Lloyd and Paul, 1929), and in syphilis (Lloyd, 1932). These authors have all along been more directly concerned with the serological control of treatment and its value in assessing the efficacy of the drug used, but incidentally they have also stressed the relative changes in the proportions of the protein constituents—albumin, total globulin, euglobulin and pseudoglobulin. The essential changes they have noticed in kala-azar and secondary syphilis consist of a fall in albumin associated with a rise in total globulin of which the euglobulin was relatively highly increased, resulting practically in the reversal of the normal globulin/albumin ratio in kala-azar, with nearly half the globulin consisting of euglobulin, while very nearly similar ratios obtained in secondary syphilis. In malaria and typhoid fever they have noticed a considerable fall in albumin with no change or a slight reduction, but not a rise, in total globulin so that while the globulin/albumin ratio was raised it never went above unity. In typhoid fever there was besides a rise in euglobulin.

Lloyd and Paul (*loc cit*) consider these protein changes essentially as immunological responses to the disease. In an individual with no resistance or where the infection is virulent the chances of obtaining the above-mentioned changes should be very slight. All the same it struck us whether we could not extend Lloyd and Paul's observations to eliciting their diagnostic value, if any, in kala-azar. With this end in view we have carried out a series of estimations of serum proteins in kala-azar, chronic malaria, typhoid fever, tuberculosis and a number of other miscellaneous conditions in cases in the senior author's wards as against controls of normal individuals consisting of his staff—altogether amounting to over 80 cases. The results have been tabulated with brief notes where necessary on individual cases (Tables I to VII). The period of investigations lasted from 22nd June, 1932 to 8th April, 1933.

DISCUSSION OF RESULTS

A study of these tables reveals certain interesting features. We must forthwith point out that the serum protein estimations are not of undoubted value in the differential diagnosis of kala-azar at every stage of the disease. At the same time we feel that in circumstances where genuine doubt arises as between kala-azar

and other diseases like chronic malaria and spleno hepatomegalies on the one hand, and kala-azar and typhoid fever and tuberculosis on the other, the serum protein values offer a certain amount of help in diagnosis short of spleen puncture. In no other disease do we find except in secondary syphilis a simultaneous rise in total globulin with increase in euglobulin. There is not much difficulty likely to arise as between kala-azar and secondary syphilis. Notable exceptions to the observations are Nos 1, 8, 10 and 11 of Table II, No 6 of Table IV, Nos 1, 5 and 6 of Table V, and Nos 2, 15, 17 and 18 of Table VII. Of these No 1 of Table II was a case of prolonged pyrexia of unknown origin with liver and spleen first becoming palpable in the course of his long stay in hospital. The case defied all attempts at diagnosis and treatment till in the end merely as a desperate measure, urea stibamine was injected and the temperature dropped to normal and remained at normal and there was rapid gain in weight and improvement in general condition. Probably he had no time to develop full immunity. Nos 8, 11 and 12 probably belong to the same class. No 6 of Table IV was a splenomegaly of unknown origin. He went home before further investigation could be made. Cases 1, 5 and 6 in Table V were all cases of undoubted well-established tuberculosis, of which No 1 presented evidences of associated kala-azar as well. In No 2 of this table, a case of acute tuberculosis which could not be labelled as such till just before death, when scanty tubercle bacilli were detected in the sputum, the changes differ widely from those found in kala-azar. It is this type of case of tuberculosis that is likely to give rise to difficulty in differential diagnosis. In Table VII, Nos 15, 17 and 18 were most likely kala-azar and they were treated as such but could not be followed up till the end as they ceased attending for injections. No 2 was a case of cirrhosis of the liver with tubercular peritonitis, as revealed by post-mortem examination.

While in themselves, then, serum protein estimations cannot establish a diagnosis of kala-azar, along with other evidences they are of real help even in cases where the formol-gel reaction is negative. Whether the serum proteins will have changed sufficiently early to permit the diagnosis with certainty of a case of very early kala-azar must be left an open question.

Interesting findings have been obtained in pernicious and other macrocytic anæmias and in a number of other miscellaneous conditions. Whether further study of the protein values in such conditions will be fruitful of valuable results it is impossible to say.

For want of accommodation, cases of kala-azar when once the diagnosis has been made are no longer kept as in-patients. They are asked to attend as out-patients for completion of the treatment. Other things being equal every adult receives as a rule 2.2 grammes of urea stibamine spread over 14 bi-weekly injections. Some of the patients drop out in the middle without completing the course, all do not attend regularly. This has been a slight handicap to the more thorough investigations of out-patients after discharge.

Attention is invited to four cases (Nos 6, 14, 15 and 16 of Table II). Case No 14 illustrates the hypothesis that active malaria does not flourish in the presence of high globulin content of blood, and that the treatment with urea stibamine brings down the globulin content to very nearly normal, when the malarial parasite can begin to proliferate and produce symptoms. Cases 6 and 15

show very nearly normal values at the completion of their course though in No 6 the gel reaction remained immediately positive. This is in accord with Lloyd and Paul's (1928) observations and suggestions that the phenomena of the gel reactions are induced by a change in the quality of the euglobulin fraction in kala-azar and not by increase in its quantitative proportion which also occurs in kala-azar, that in the course of treatment the return of euglobulin to normal value is quicker than the restoration of its original quality. Case 16 attracts special notice because of the simultaneous activity of both malarial and kala-azar parasites and of the former in presence of high globulin induced by the latter. In fact we had no reason to think of kala-azar in the case till with the persistence of fever in spite of the disappearance of malarial parasites from peripheral blood on exhibition of atabrin, we did a 'gel' test to exclude kala-azar. To our surprise the test was immediately a strong positive and to complete the diagnosis we found Leishman-Donovan bodies in the material obtained after splenic puncture. The patient was responding well to urea stibamine. Unfortunately she would not stay for completion of treatment and she did not attend for injections after discharge. We had no opportunity of judging whether the malarial parasites had been exterminated or whether they reasserted themselves under more favourable conditions brought about by return of globulin to normal values with treatment for kala-azar. We do not know whether a similar case has been recorded. Details of this case will be published elsewhere.

SUMMARY AND CONCLUSIONS

1 The serum protein changes in kala-azar while not absolutely diagnostic of the disease offer distinct help in diagnosis of kala-azar in doubtful conditions.

2 The essential changes in serum proteins in kala-azar are a fall in albumin with considerable rise in total globulin, associated with increase in the relative proportion of euglobulin. A similar, but less striking change, has previously been recorded by Lloyd in secondary syphilis.

3 Serum protein changes in a number of miscellaneous conditions have been recorded.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the hearty co-operation we have received from Dr A S Mannadi Nayar, Professor of Biochemistry, Medical College, Madras, and for lending the services of his assistant for this investigation. On account of retrenchment the Biochemist's staff was curtailed and yet he readily agreed to do the biochemical investigations for us. Appended is a note (by A S R) on the methods used for the determination of serum proteins.

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APPENDIX

METHODS FOR THE DETERMINATION OF ALBUMIN, GLOBULIN AND EUGLOBULIN IN BLOOD SERUM

Two colorimetric methods were employed —

- (1) Wu's method using Wu's phenol reagent (Cole, 1926)
- (2) Wu and Ling's is modified by Greenberg, using Folin and Ciocalteu's phenol reagent (Greenberg, 1929)

Both the methods were slightly modified to suit the conditions of our laboratory

The first method

The precipitation of the protein fractions is done by the ammonium sulphate method. To the first of three test tubes in a rack 9.5 c.c. of 0.8 per cent NaCl is added and then 0.5 c.c. of blood serum. From the contents of this tube the total proteins are estimated. To the second tube 9.5 c.c. of 5/9.5 saturated ammonium sulphate is added and then 0.5 c.c. of blood serum effecting a half saturated ammonium sulphate. This precipitates the globulins. To the third test-tube 9.5 c.c. of 3.3/9.5 of saturated ammonium sulphate is added and then 0.5 c.c. of blood serum to bring about one-third saturation. This precipitates the euglobulins. One drop of toluol is added to each of the three test-tubes and they are kept at laboratory temperature overnight. Next morning the contents of the second and third tubes are filtered through No. 50 Whatman filter-paper. Two c.c. of the contents of the first tube and 2 c.c. of the filtrates from the second and third test-tubes are transferred to three centrifuge tubes and about 8 c.c. of water is added to each and then 1 c.c. of 10 per cent sodium tungstate, and 1 c.c. of $2/3 \text{ NH}_2\text{SO}_4$. After 15 minutes the tubes are centrifuged for about 10 minutes at 2,000 revolutions per minute, the supernatant fluid is removed from all the tubes, and the contents transferred with a little water to three 25 c.c. flasks and two drops of 20 per cent Na_2CO_3 is added to each and shaken and then about 15 c.c. of water. The standard is prepared by adding 1 c.c. of tyrosine solution (1 c.c. = 0.2 mg. tyrosine) to a 25 c.c. flask containing about 20 c.c. of water. To all the four flasks 0.5 c.c. of Wu's phenol reagent is added, and then with constant shaking 3 c.c. of 20 per cent Na_2CO_3 . The flasks are filled up to the mark with water, shaken well and after 30 minutes their colours are compared in a colorimeter setting the standard at 20. The first flask will give the tyrosine equivalent to the total proteins, the second albumin, the third albumin and pseudoglobulin. From the results obtained the albumin, globulin and euglobulin content of the serum is worked out.

The second method

The major portion of the determinations were done by this method which is more accurate and quicker. The precipitation of the protein fractions is done by Howe's (1921) method. To the first of three test-tubes in a rack 9.5 c.c. of distilled water is added and 0.5 c.c. of blood serum. From the contents of this tube, the total proteins are estimated. To the second test-tube 9.5 c.c. of 22.5 [per cent anhydrous Na_2SO_4 is added and then 0.5 c.c. of blood serum. This precipitates

the serum globulins To the third test-tube 9.5 c.c. of 14.5 per cent anhydrous Na_2SO_4 is added and then 0.5 c.c. of blood serum This precipitates the euglobulins One drop of toluol is added to each of the tubes and they are kept at room temperature overnight The contents of the second and third tubes are filtered next morning through No. 50 Whatman filter-paper and 5 c.c. of the contents of the first tube well shaken, and 5 c.c. of the filtrates from the second and third tubes are transferred to three 50 c.c. flasks and about 30 c.c. of water is added to each Four c.c. of standard tyrosine solution (1 c.c. = 0.2 mg tyrosine) is added to another 50 c.c. flask containing about 30 c.c. of water Two c.c. of 5 N NaOH is added to each of the four flasks and then 3 c.c. of Folin and Ciocalteu's (1927) phenol reagent with constant shaking The flasks are filled up to the mark with distilled water Their colours are compared in a colorimeter after 30 minutes the standard being set at 20 The tyrosine equivalents of total proteins, albumin, and albumin and pseudoglobulin fractions are worked out from the colorimeter readings and from a knowledge of the tyrosine values for the protein fractions of the human serum, the albumin, globulin and euglobulin values are worked out

The colorimetric method is said to be liable to errors up to about 10 per cent (Peters and van Slyke 1931) Since many normal cases are done by the same method, the results will give an accurate comparative idea of the amount of the protein fractions of the blood serum in kala-azar

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TABLE I
Normal serum protein values

Case No	Total serum protein per cent	Serum albumin per cent	Serum globulin per cent	Serum englobulin per cent	Serum pseudo globulin per cent	Globulin Albumin ratio	I uglobulin Globulin per cent	REMARKS
1	5.550	1.100	1.150	0.350	0.800	0.26	30	Vegitarian
2	7.069	5.013	2.656	0.252	2.404	0.53	9	Non vegetarian
3	5.893	4.235	1.658	0.504	1.154	0.39	30	do
4	6.570	4.250	2.320	0.562	1.758	0.55	24	do
5	5.486	3.528	1.958	0.590	1.308	0.55	30	do
6	5.790	3.680	2.110	0.490	1.620	0.57	23	do
7	5.930	3.640	2.290	0.690	1.600	0.63	30	Vegetarian
8	5.777	3.353	2.424	1.257	0.977	0.61	57	Vegetarian after a meal
9	6.455	3.840	2.615	0.786	1.829	0.68	30	Vegetarian
AVERAGES	6.195	4.019	2.106	0.600	1.497	0.52	29	
Lloyd and Paul's averages of ten Indians	7.622	4.592	2.930	0.166	1.764	0.64	6	Vide Ind Jour Med Res, 16 No 2 October, 1928 p 529

TABLE II

Serum protein values in kala-azar

Case No	Total serum protein per cent	Serum albumin per cent	Serum globulin per cent	Serum euglobulin per cent	Serum p-endo globulin per cent	Globulin ratio Albumin	Euglobulin Globulin per cent	REMARKS
1	6.687	3.135	3.552	0.932	2.620	1.13	26	Gel negative Prolonged fever Immediate response to urea stibamine Spleen and liver just palpable
2	7.79	2.750	5.040	2.835	2.205	1.83	56	Gel +
3	6.264	2.217	4.047	1.348	2.609	1.83	33	Gel - R B C W B C +
4	5.662	1.999	3.663	2.696	0.967	1.83	74	Gel + R B C W B C +
5	6.497	2.357	4.140	2.966	1.174	1.76	72	
6	7.037	2.206	4.831	4.185	0.466	2.20	91	Spleen puncture +, +, +, Gel + + +, After full course, Alb 2.558, 2.039 Glob, Eu 0.65 but Gel + + + + 3 ⁺
7	6.030	2.585	3.445	3.188	0.277	1.33	93	Gel - R B C W B C +
8	4.651	2.200	2.361	0.599	1.762	1.03	24	Jaundice + +, Gel + +, R B C W B C + Treated with neo-stibosan

9	5 331	1 413	3 018	3 044	0 881	2 77	77	Marked leucopenia Spleen + + +, Response to U S +
10	6 381	1 020	2 301	2 042	0 110	0 79	80	Gel + Not complete Response only to U S, Spleen +, Liver +
11	4 001	2 819	2 172	0 905	1 207	0 77	15	Perisplenitis R B C W B C → Double rise
12	4 036	1 066	2 970	1 525	1 115	1 78	52	Cel + R B C W B C + (antrum only)
13	4 033	1 004	3 029	1 618	1 381	1 59	55	Spleen puncture + +
14	5 818	2 675	3 113	1 581	1 502	1 18	50	Gel + Had a full course of uret stibamine (came five months later with malaria V 1 and a different protein content of albumin globulin 1.31, 2.019 Euglobulin was 1.280 out of 2.018 globulin
15	7 757	2 088	5 009	2 522	2 517	1 88	50	Perisplenitis No mentation Had urea stibamine later See No 22
16	6 823	2 315	4 488	2 301	2 197	1 92	51	Spleen puncture + + +, Gel + + + No mentation Malarial parasites in peripheral blood film, treated with atebrin and then because of persistence of fever gel test and spleen puncture were done

TABLE II—*concl'd*

Case No	Total serum protein per cent	Serum albumin per cent	Serum globulin per cent	Serum euglobulin per cent	Serum pseudo globulin per cent	Globulin ratio	Euglobulin Globulin per cent	REMARKS
17	7.666	1.646	6.020	3.669	2.351	3.66	61	Gel +++ Slight jaundice
18	7.521	2.240	5.281	2.812	2.469	2.36	53	Gel ++
19	5.276	2.444	2.832	1.514	1.318	1.16	53	Gel negative R B C W B C + Came from Bangalore with seven months fever not amenable to neostibosan (25 injections) Response to urea stibamine prompt
20	5.610	2.622	2.988	1.548	1.440	1.14	52	Gel - R B C W B C +
21	5.836	2.906	2.930	1.389	1.541	1.01	47	Gel - Response to urea stibamine + after failure of other treatment
22	7.711	2.757	4.954	3.222	1.732	1.80	65	Gel doubtful (same as No 15) After full course—Alb 2.987, Glob 2.861, Euglob 1.581 Patient had had homöopathic treatment and 0.15 g of urea stibamine

23	6 20	1 790	1 410	2 400	2 010	2 46	51	Gel ++
24	5 27	1 710	3 530	1 080	1 850	2 03	18	Gel ++
25	6 578	2 906	3 052	1 073	1 699	1 26	53	Gel ++ No pigmenta- tion Had 'typhoid', two months back Splenic ++
26	5 781	1 519	1 202	2 713	1 519	2 81	61	Gel ++ Carcinoma
27	5 101	2 623	2 478	1 234	1 224	0 95	50	Treble rise P B C W B C + No re- sponse to prolonged quinine Response to urea stibamine im- mune date
28	5 755	2 386	3 369	2 678	0 091	1 41	80	Gel ++ Treble rise
Average for kala azar—28 cases	6 128	2 380	3 748	2 193	1 555	1 57	58.5	
Lloyd and Paul (average of 8 cases of kala azar)	7 645	3 202	4 413	2 050	2 393	1 39	46	Vide <i>Ind Jour Med Res</i> , 16, No 2 October, 1928, p 529
Lloyd (average of 11 cases of secondary syphilis)	7 279	3 313	3 966	1 470	2 496	1 20	37	Vide <i>Ind Jour Med Res</i> 19, No 4, April, 1932, p 1055

TABLE III
Serum protein values in typhoid fever

Case No	Total serum protein per cent	Serum albumin per cent	Serum globulin per cent	Serum euglobulin per cent	Serum pseudo-globulin per cent	Globulin $\frac{\text{ratio}}{\text{Albumin}}$	Euglobulin $\frac{\text{Globulin}}{\text{per cent}}$	REMARKS
1	5.992	3.437	2.555	1.343	1.212	0.74	52	Blood culture +
2	6.787	4.229	2.558	1.898	0.660	0.61	74	do
3	7.248	3.740	3.508	3.155	0.353	0.94	90	do
4	4.955	2.896	2.059	0.980	1.079	0.71	48	do
5	5.125	2.856	2.269	0.469	1.800	0.80	21	do
6	5.501	3.586	1.915	1.411	0.504	0.53	73	Culture + Protein estimation done on the day of discharge

TABLE IV
Serum protein values in chronic malaria and other splenomegalies—probably malarial

Case No	Total serum protein per cent	Serum albumin per cent	Serum globulin per cent	Serum onoglobulin per cent	Serum pseudo globulin per cent	Globulin ratio Albumin	Gamma globulin per cent	REMARKS
1	6.288	3.530	2.738	1.814	0.924	0.77	66	Spleen ++ Liver + 1 and 3 ever on alternate days with rigor
2	5.501	3.217	2.374	1.638	0.736	0.71	69	Spleen puncture showed no Leishman Donovan bodies Gel \pm Spleen ++ hard Liver +
3	5.311	3.245	2.066	1.222	0.814	0.64	39	Spleen puncture showed no Leishman Donovan bodies
4	4.563	2.622	1.941	0.480	1.161	0.74	25	Spleen ++ Liver + Spleen puncture negative for Leishman Donovan bodies (done twice)
5	5.631	3.583	2.048	1.280	0.769	0.57	63	M J runs and crescents Had had a full course of urea stibamine five months back Globulin high then
6	6.386	3.074	3.312	1.339	1.973	1.08	10	Spleen ++ Was in F M S Gel -
7	7.092	3.578	3.514	1.598	1.916	0.98	46	Spleen ++ hard Antenna ++ Gel - Kahn and Wassermann +++
8	4.510	2.906	1.604	0.432	1.172	0.55	27	Spleen ++, Liver ++, Gel - No response to quinine Had only one urea stibamine

TABLE V
Serum protein in values in pulmonary tuberculosis

Case No	Total serum protein per cent	Serum albumin per cent	Serum globulin per cent	Serum englobulin per cent	Serum pseudo globulin per cent	Globulin/Albumin ratio	Englobulin/Globulin per cent	REMARKS
1	5.668	2.271	3.397	2.958	0.439	1.49	87	Fibrosis lump Had had 20 A P's Liver and spleen + ? K A
2	6.197	3.877	2.320	0.294	2.026	0.60	13	Acute T P Died No P M Sputum +
3	5.216	2.481	2.735	0.470	2.265	1.10	17	Active T P Sputum +
4	4.753	2.528	2.225	0.832	1.373	0.73	38	Active T P Spleen + No parasites in blood Died in hospital Sputum +
5	6.296	2.970	3.326	1.453	1.843	1.12	45	T P Sputum +
6	6.125	2.360	3.565	1.569	1.996	1.39	44	Active T P with occasional hemoptysis Sputum +

TABLE VI
Serum protein values in pernicious and other macrocytic anemias

Case No	Total serum protein per cent	Serum albumin per cent	Serum globulin per cent	Serum euglobulin per cent	Serum pseudo globulin per cent	Globulin/Albumin ratio	Iuglobulin/Globulin per cent	Remarks
1	2.505	0.791	1.811	0.880	0.931	2.29	49	Pernicious anemia
2	4.096	2.819	1.877	0.966	0.911	0.67	51	do
3	5.785	1.259	2.126	0.728	1.598	0.72	31	Sprue
4	5.153	3.811	1.349	0.412	0.097	0.35	12	do
5	4.518	2.141	2.091	1.118	0.976	0.86	51	Pernicious anemia
6	6.053	3.259	2.794	1.197	1.397	0.86	50	Pernicious anemia with S C D
7	5.832	3.126	2.506	1.512	0.991	0.75	60	Sprue
8	6.046	3.167	2.579	1.362	1.217	0.71	53	Hemolytic macrocytic anemia of undefined origin Spleen ++

TABLE VII

Serum protein values in miscellaneous conditions

Case No	Total serum protein per cent	Serum albumin per cent	Serum globulin per cent	Serum euglobulin per cent	Serum pseudo-globulin per cent	Globulin ratio	Euglobulin/Globulin per cent	REMARKS
1	5.713	3.750	1.963	1.696	0.267	0.52	87	Had had V D and under gone anti syphilitic course with development of jaundice Spleen puncture (done twice) negative Later had two empirical urca stibamine injections Later still came back in a collapsed condition after profuse bleeding per rectum Oscillating van den Bergh Spleen ++, upwards Liver +, Gel - Lung fields clear
2	5.630	2.750	2.880	1.986	0.894	1.05	69	Spleen + + +, Gel -, Ascites + Post-mortem, cirrhosis liver with tubercular peritonsils
3	6.123	3.666	2.457	2.112	0.345	0.67	86	W R + + +, Gel - Spleen puncture showed plasma cells Liver and spleen + Pigmentation face Reddish patches on palms

4	3 776	1 944	1 832	0 875	0 977	0 94	19	Liver + + jaundice +, Ascites + Died of choleemia in hospital
5	5 328	2 211	3 117	1 616	1 471	1 12	73	Spleen called sarcoma lune (? metastatic)— pathologist's report on island section Died in hospital No post mortem
6	6 416	4 400	2 016	0 479	1 737	0 46	24	P U O enteric in type
7	5 786	4 630	1 156	0 360	0 796	0 25	31	Myeloid leucemia
8	4 087	2 437	1 650	1 069	0 581	0 68	65	do
9	5 302	3 162	2 140	0 962	1 178	0 68	15	do
10	5 551	3 553	1 968	0 544	1 424	0 55	28	Diabetes mellitus
11	5 353	3 259	2 094	0 880	1 214	0 61	12	A complete calcification of the descending thoracic aorta asso- ciated with cardiac syphilis Death in a few days after dis- charge
12	5 848	2 087	2 961	1 584	1 280	0 96	55	K A after completion of course (22 g of urea stibumme)

TABLE VII—*concl'd*

Case No	Total serum protein per cent	Serum albumin per cent	Serum globulin per cent	Serum euglobulin per cent	Serum pseudo globulin per cent	Globulin Albumin ratio	Euglobulin Globulin per cent	REMARKS
13	4.597	2.558	2.039	0.650	1.389	0.80	32	K A after completion of 22 g of urea stibamine Gel+++ ³
14	6.400	3.780	2.620	1.660	0.960	0.69	63	
15	6.364	2.922	3.442	1.220	2.222	1.18	36	Splenomegaly of unknown origin Gel — General pigmentation
16	5.484	2.560	2.924	0.773	2.151	1.14	27	
17	6.783	2.688	4.095	2.376	1.719	1.52	58	Had had previous incomplete treatment for kala azar
18	1.882	0.894	0.988	0.714	0.274	1.12	36	

STUDIES ON THE ANTIGENIC STRUCTURE OF *VIBRIO CHOLERÆ*

Part III

FURTHER ANALYSES OF SPECIFIC CARBOHYDRATES

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ON the basis of our previous work (Linton, 1932 , Linton and Shrivastava, 1933a, 1933b), we have put forward the hypothesis that in the vibrios, derived either from water or from clinical cholera, two types of specific carbohydrate are present. We have shown that these two substances are similar in structure , in both, the aldobionic acid portion consists of galactose and glycuronic acid, and to this complex is attached in some strains a molecule of arabinose and in other strains a molecule of galactose. Our earlier work led us to believe that these two carbohydrates were structures whereby the water-vibrios might be distinguished from the pathogens in that the water-vibrios were characterized by the possession of the arabinose-containing carbohydrate and the pathogenic forms by the galactose-containing substance.

As will be shown in this paper we cannot make this distinction, however, since we have now found two strains from clinical cases of cholera in which the specific carbohydrate has been arabinose-containing. This finding appears to be of some importance in tending to establish a close relationship between water-vibrios and those which occur in cholera , especially as the typical non-agglutinating water-vibrios which we have examined have possessed the arabinose-containing carbohydrate.

In this paper we are presenting analyses of six vibrios two isolated from water, and four from cases of cholera. The carbohydrates of Nos. W25, 505, 1612 and 585 have been prepared by the methods previously described. The carbohydrates of the other two strains, W3075 and 1687, have been obtained by another method designed to avoid any possible contamination of the vibrio carbohydrate with carbohydrate which might have come in from the agar on which the organisms are grown. Essentially, the vibrios are freed as completely as possible of the fluid in which their growth has occurred.

The method which closely follows one described by Furth and Landsteiner (1928), may be briefly outlined. The organisms are washed off in sterile distilled water containing phenol to make a final dilution of 0.5 per cent. The washings are immediately put through the Sharples Supercentrifuge, taken up in distilled water, thoroughly mixed, and centrifuged again. The process is repeated. The final wash water is faintly yellow and slightly opalescent.

The vibrios are now dissolved in $N/2$ NaOH and the solution allowed to stand in the incubator at 37°C for about an hour. Hydrochloric acid is then added until the solution is only slightly alkaline, insoluble matter is centrifuged off, and the solution precipitated with $1\frac{1}{2}$ volumes of absolute alcohol. After standing overnight in the refrigerator the precipitate is taken up in water, insoluble matter removed by centrifuging, and hydrochloric acid added to make a $N/10$ solution. The heavy protein precipitate is removed in the centrifuge, and the supernatant fluid, after being made alkaline, is again precipitated with $1\frac{1}{2}$ volumes of alcohol. The precipitate does not give any biuret reaction and does not reduce Benedict's solution, except after hydrolysis.

The yield of carbohydrate by this method is small but satisfactory analyses have been possible.

RESULTS

Water-vibrio No. 25—A smooth non-agglutinating vibrio, received from the Pasteur Institute, Shillong, and originally derived from water in Shillong. 9.8 g of the gum were mixed with 1 l sulphuric acid and placed in the incubator at 37°C for 42 hours. The amount of reducing substance obtained was 17.9 per cent, calculated as glucose. The hydrolysate was fractionated as previously described, in some experiments Fraction II was removed with alcohol, and in others as the calcium salt. When heated for 12 hours on the water-bath at 80°C the reducing substances in Fraction II increased by 48 per cent. No further increase could be obtained after heating for 6 hours longer.

To a portion of the hydrolysed Fraction II barium carbonate was added, and the resulting salt, which was presumably barium glycuronate, was decomposed, and the decomposition product reduced Benedict's solution in the cold, a phenomenon which is characteristic of glycuronic acid. The supernatant fluid, from which the barium salt had been precipitated, was treated with phenylhydrazine. Two types of crystals appeared, the first of which melted at 185°C and the second at 202°C . Since the material in solution had been precipitated only once with barium it is probable that the higher melting point was that of the phenylosazone of glycuronic acid, which had been left behind. The known melting point of this compound is 200°C – 203°C (Noid 1922).

Fraction III give the following compounds and melting points —

Phenyllosazone 160°C–162°C, 157°C–158°C Known arabinose phenyllosazone melts at 160°C

Methylphenylhydrozone 160°C–161°C The known melting points of the arabinose compound are given by Zemplen (1922) as 161°C and 164°C

Mucic acid was formed and melted at 218°C–220°C Potassium acid saccharate was also formed

We may conclude that the carbohydrate of water-vibrio No 25 has the same structure as that of other water-vibrios previously studied It consists of an aldobionic acid and arabinose The acid itself is composed of galactose and glucuronic acid

Vibro No 505 —This organism was received from Dr J Basu of the Bengal Vaccine Laboratory Calcutta It is a smooth agglutinating vibrio, isolated early in a clinical case of cholera

10.9 g of the polysaccharide were kept for 42 hours at 37°C with 1.1 sulphuric acid, and at the end of this period yielded 19.5 per cent of reducing substances Fraction II was twice separated as the calcium salt, which on decomposition showed 0.375 g of reducing substance, calculated as glucose Hydrolysis on the steam-bath gave the following increases in reducing substance at the times noted —

Period of heating	Amount of reducing substance as glucose g	Percentage increase
Original	0.375	
11 hours	0.700	86
13	0.788	110
15 "	0.806	114
17	0.825	120
19 "	0.806	114

Other workers have found that the maximum hydrolysis of aldobionic acids occurs after about 18 hours' heating It is probable that the fall in amount at 19 hours represents destruction of the reducing substance

The hydrolysate of Fraction II was concentrated to dryness, taken up in hot acetic acid, seeded with galactose and placed in the refrigerator for one week. The crystals which appeared melted at 156°C–160°C. On re-crystallization their melting point was 167°C. Known galactose also melted at 167°C.

The mother liquor remaining after filtering off the crystals was evaporated to dryness *in vacuo* to get rid of acetic acid. The dried material, taken up in a little water, reduced Benedict's solution in the cold, thus showing the presence of glycuronic acid.

The following compounds were formed from Fraction III, and their melting points determined —

Phenylosazone m p 163°C–165°C, 160°C–163°C. Known arabinose phenylosazone melts at 160°C.

Methylphenylhydrozone m p 169°C. The known arabinose compound melts at 164°C.

Here again Fraction II consists of glycuronic acid and galactose, while Fraction III is pretty clearly arabinose. This organism, then, is an example of a strain containing arabinose, and which is at the same time an agglutinating vibrio isolated from a case of cholera.

Vibrio 1612 — This organism, which was received from Captain C. L. Pasricha, I. M. S., was isolated from a severe case of cholera on the second day of the disease, the patient died on the third day. It is a rough, non-agglutinating vibrio, and was the only vibrio isolated from this case.

A portion of the gum (7.76 g) was hydrolysed on the sand-bath for 2 hours and yielded 22 per cent of reducing substances, calculated as glucose. The aldobionic acid was removed as the barium salt, and Fraction III was treated in the way already described for the crystallization of galactose. After standing for 8 days in the refrigerator, the snowy white crystals were filtered off, and dried. They melted at 167°C, reduced Benedict's solution strongly, had a sweet taste and showed a specific rotation of +87.4° (285 mg in 10 c.c. water).

The filtrate from these crystals yielded a phenylosazone which melted at 199°C. Known glycuronic acid phenylosazone melts at 200°C–203°C.

An attempt to crystallize arabinose from the filtrate gave a negative result.

A second portion of the gum (9.53 g) was heated on the water-bath for 30 hours at 80°C, and gave 20.8 per cent hydrolysis. The aldobionic acid was again precipitated with barium, and Fraction III treated as before and seeded with galactose. After a week's time the crystals which had appeared had a melting point of 154°C–155°C and a specific rotation of +87.5° (169 mg in 10 c.c. of water). On re-crystallization the sugar melted at 160°C.

An attempt to crystallize arabinose from this material also gave a negative result.

The polysaccharide of this vibrio has the usual aldobionic acid structure in Fraction II, and Fraction III consists of galactose. This vibrio is particularly interesting, because, although it is rough and non-agglutinating it still possesses

the same polysaccharide as is found in some smooth and agglutinating organisms

In our previous work we have given an instance of a rough non-agglutinating organism having the same carbohydrate structure as the smooth, agglutinating form from which it was derived. In that instance, at least, the smooth-rough transition had no effect upon the polysaccharide.

Vibrio 585 — This strain was obtained from the Pasteur Institute, Shillong, and had been derived originally from a fatal case of cholera in Karachi in 1932. It agglutinates at a low titre with Kasauli cholera serum, and is morphologically a typical vibrio.

The gum derived from this vibrio (5.848 g) was heated with acid for 3 hours on the water-bath at 80°C, and then put for 16 hours in the incubator at 37°C. The yield of reducing substances was 29.9 per cent calculated as glucose. Fraction II was twice precipitated as the barium salt, and on treating a portion of it with nitric acid mucic acid was formed which melted at 222°C. Potassium acid saccharate was also formed from the same portion, showing thus the presence of glycuronic acid. The sugar which crystallized out of Fraction II, after seeding with galactose, weighed 0.3182 g, melted at 160°C–161°C, and had a specific rotation of +78.9°C (0.114 g in 10 c.c. of water).

Fraction III gave mucic acid which melted at 224°C.

Vibrio 1687 — This smooth agglutinating strain, received from Captain Pasricha, was isolated from a severe case of cholera, which terminated in death.

The carbohydrate (0.220 g) was hydrolysed for 8 hours at 80°C on the water-bath and gave 12.4 per cent of reducing substances. A phenylosazone formed from Fraction III melted sharply at 182°C, and the appearance of the crystals under the microscope was that of galactose. Fraction II yielded potassium acid saccharate, thus showing the presence of glycuronic acid.

The results of the study of vibrio 1687 indicate that we are dealing with an organism having galactose as the characteristic sugar attached to the aldobionic acid.

Water-vibrio No 3075 — This non-agglutinating water-vibrio was received from Captain Pasricha. It has the smooth-rough type of growth, as shown by testing with Millon's reagent.

We have isolated from it a sugar whose phenylosazone melted at 158°C, and an aldobionic acid which showed the presence of galactose and glycuronic acid. This organism then resembles other water-vibrios in that it is characterized by the possession of arabinose in its specific substance.

DISCUSSION

In this paper and the preceding one, we have presented analyses of ten vibrios. Their sources, the sugars present in the Fraction III of each one, and their reaction to agglutinating serum may be seen in the table below. The composition of

Fraction II has not been indicated, in every vibrio in the table it has been found to consist of galactose and glycuronic acid

TABLE

Number	Origin	Sugar found in Fraction III	Agglutination reaction
454 Smooth	Cholera	Galactose	Agglutinable
454 Rough	454 Smooth	,	Inagglutinable
1612	Cholera	„	Agglutinable
585	„	,	,
1697	„	„	„
505	„	Arabinose	„
E	„	„	„
W841	Water	„	Inagglutinable
W25	„	„	„
W3075	„	,	„

As we have already pointed out, these vibrios are divisible into two groups, one of which contains galactose and the other arabinose. With two exceptions the vibrios derived from cholera cases are galactose-containing, and all the water-vibrios contain arabinose. However, strains 505 and E show that this is no absolute rule, since both were derived from cases of cholera and both possess an arabinose-containing polysaccharide. The bearing of this finding on the question of the relationship between water-vibrios and clinical cholera is highly suggestive.

SUMMARY

Analyses are given of the specific carbohydrates of six vibrios, four of them derived from cholera cases and two from water. The previous finding of two types of specific substance in the vibrios is extended to these strains, some of which contain galactose and some arabinose as the characteristic part of their polysaccharides.

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STUDIES ON THE ANTIGENIC STRUCTURE OF *VIBRIO CHOLERÆ*

Part IV

A PRELIMINARY EXAMINATION OF CARBOHYDRATES IN THE RICE-WATER STOOLS OF CHOLERA PATIENTS

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METHOD

THE stools which we have examined were collected at intervals of a few hours from patients in the Cholera Wards of the Campbell Hospital, Calcutta. Only fluid stools, white to light yellow in colour, were taken and semi-solid stools and those stained with blood were not used. The stools, which were usually alkaline, were made slightly acid to litmus with acetic acid, and concentrated on the water-bath. Between one and three litres of stool was obtained from each case, and this amount was reduced in volume to between 200 c c and 400 c c. The heavy precipitate which appeared during the concentration was centrifuged off, and strong NaOH added slowly and with constant stirring until the fluid was just alkaline. During the addition of the alkali a heavy flocculum separated out and was removed in the centrifuge. The clear supernatant fluid was then precipitated with one and one-half volumes of absolute alcohol, and the white flocculum which appeared during the course of an hour's standing was collected and dried in the hot-air oven. This substance does not give a biuret reaction and its polysaccharide nature is shown by the fact that it does not reduce Benedict's solution until it has been hydrolysed, after which it reduces strongly.

The dried carbohydrate was weighed, and dissolved in 3 per cent H_2SO_4 . The amount of acid used for dissolving varied from 10 c c to 50 c c according to

the weight of the substance. The solution was placed under reflux in the boiling water-bath for 2 or 3 hours, and at the end of that time reducing substances had appeared in amounts sufficient to give a strong reduction with Benedict's solution. The hydrollysate was then treated with sodium acetate until it was neutral to congo red paper, and one volume of alcohol was added to remove the inorganic salts. After centrifuging, 0.3 c.c. to 0.8 c.c. of phenylhydrazine mixture (1 part phenylhydrazine and 1 part of 50 per cent acetic acid) was added to the clear supernatant fluid, and the tube placed in the boiling water-bath for 30 or 40 minutes. The crystals which appeared after allowing the solution to cool in the water-bath (and, preferably, to stand overnight) were filtered off, purified by dissolving in boiling water, allowed to re-crystallize, dried, and the melting point determined in the usual way.

By this method it is possible to identify a sugar derived from a polysaccharide in a stool within a few hours after receiving the stool in the laboratory.

RESULTS

Details of the eleven stools examined by the above method are shown in the following table. The amount both of polysaccharide and of reducing substance derived from it are quite variable. We cannot say whether this variation is an actual result of the disease state, or whether deficiencies in the technique are responsible. It is probable that further effort will give us more consistent results on this point, but it should be emphasized that as it stands the method is adequate for the isolation and identification of sugars in rice-water stools.

TABLE

Analysis of polysaccharides derived from rice-water stools of patients infected with cholera

Number	Amount of stool c.c.	Yield of polysaccharide mg.	Percentage hydrolysis	Melting point of phenylhydrazine compound °C.	Outcome of the infection
1*	1,500			183	
2*	2,000	260	8	183-185, 185	Recovered
3*	1,500			180	Died
4	1,000	460	6	184-185	Recovered
5	2,500	700	17	180	Do
6	1,300	1,510	1	178	Died
7	2,600	970	12	179-181	Recovered
8	2,750	2,900	6	166-168, 155	Do
9*	1,000			166	Died
10	2,000	900	3	162	Do
11	1,000	240	3	135, 135	Recovered

* These polysaccharides were extracted from the stools by the method previously described (Linton and Shrivastava, 1933b).

The melting points of the phenylosazones (with the exception of No 11, which we shall discuss below) may be divided into two groups. In the first are those melting at temperatures varying from 162°C to 168°C (Nos 8, 9 and 10), and second the remaining sugars, whose osazone compounds melt between 178°C and 185°C and average 182°C for the set of observations.

It appears to us suggestive that the melting points of these two groups approximate those of the phenylosazone compounds of arabinose (160°C) and galactose (186°C). These are the sugars which we have previously found to be characteristic for the two polysaccharides derived from the vibrios, when they have been grown *in vitro* (Linton and Shrivastava, 1933a, 1933b). That these sugars should appear in cholera stools is not surprising, and confirms to a certain extent our previous findings.

It is of interest to note that the stools numbered 8, 9 and 10 may contain arabinose, the sugar which has also been found in the water-vibrios so far investigated, and that the first two of these cases ended fatally. We have already reported the presence of two other arabinose-containing vibrios in cultures isolated from cholera cases. It appears therefore that both types—galactose as well as arabinose-containing vibrios—may occur in the disease, although we are not yet in a position to state whether both are causative agents of cholera. It should be noted that both the arabinose-containing vibrios previously studied were agglutinable, and that the vibrio isolated from stool No 9 was also agglutinable. We do not have a record of the agglutination reaction of vibrios from the other two stools.

Stool No 11 is of some interest. The melting point of 135°C found does not correspond to that of any known phenylosazone. We therefore prepared mixtures of known arabinose and galactose in the following proportions, formed the phenylosazones, and determined the melting points—

Arabinose Per cent	Galactose Per cent	Melting point °C
25	75	172–182
50	50	135
75	25	130

A second sample, where the proportions were estimated by eye and not by weighing as above, gave a phenylosazone melting at 137°C for a mixture of equal parts of the two sugars.

It is possible therefore that in this case both types of organism were present in approximately equal numbers, and each yielded an equal amount of its characteristic sugar. We do not, however, claim that this interpretation is the correct one, nor, since it is a single example, are we able to lay any stress upon it.

We have made an attempt to separate the hydrolysed polysaccharides into fractions similar to those upon which we have reported in our previous work. We were not successful, however, in identifying the constituents of these fractions (particularly those of the aldobionic acid described previously in Fraction II) probably because of the minute amounts of material available for analysis when the

AN INVESTIGATION INTO 'DECOMPENSATED PORTAL CIRRHOSIS'

BY

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It was pointed out in previous communications (Radhakrishna Rao, 1932, Tirumurti and Radhakrishna Rao, 1932) that the results of the investigation—both clinical and pathological—into the causation of ascites in this part of India, definitely show that decompensation of the heart and cirrhosis of the liver are the commonest causes of ascites and that many of the cirrhosis of the liver are of the portal type. In the present paper, cases of 'Decompensated Portal Cirrhosis' are studied in greater detail with the object of throwing further light on the subject. Many patients suffering from 'decompensated portal cirrhosis' are admitted every year into the Medical Wards of the King George Hospital, Vizagapatam, and my observations and the results of investigations on these patients during the last two years are given in this paper.

(1) *Selection of cases*

Only those cases with definite evidences of portal obstruction and ascites were selected for this study. In all these cases the diagnosis was confirmed after a thorough investigation. Out of the 64 cases selected for this study the clinical diagnosis was verified at operation in 2, and at necropsy in 12 cases.

(2) *Incidence*

In a series of 86 cases of cirrhosis of the liver (excluding the cases of infantile biliary cirrhosis) carefully investigated in the Medical Wards of the King George Hospital, Vizagapatam, 65 cases were associated with ascites, while in the rest

(21 cases) there was no fluid in the abdomen. The causes of cirrhosis of the liver in the 21 cases without ascites are given in Table I below —

TABLE I
Showing the causes of cirrhosis in the 21 cases without ascites

Serial number	Cause of cirrhosis	Number of cases
1	'Gummatous hepatitis' (<i>Hepar lobatum</i>)	1
2	Syphilitic cirrhosis	1*
3	Late congenital syphilis	5
4	Splenic anaemia	2†
5	Bronzed diabetes (?)	1
6	Portal cirrhosis	7‡
7	Hypertrophic biliary cirrhosis	4

* In this case there was marked improvement after anti syphilitic treatment

† The clinical diagnosis was verified at necropsy in one case

‡ The clinical diagnosis was verified at necropsy in two cases, two cases were admitted for bleeding piles and one gave a history of repeated hæmatemesis

Following Rowntree's revised clinical classification of cirrhosis of the liver (*cf* Chapman, Snell and Rowntree, 1931), the present series of cases is classified as follows (Table II) —

TABLE II
Showing an analysis of the present series of cases based on Rowntree's clinical classification of cirrhosis of the liver

	(A) Compensated cirrhosis (with adequate portal circulation) Number of cases	(B) Decompensated cirrhosis with ascites (inadequate portal circulation) Number of cases
1 Portal cirrhosis (large or small liver)	7*	64
2 Biliary cirrhosis—		
(a) without extrahepatic biliary obstruction	4*	1
(b) with extrahepatic biliary obstruction		

* These cases are taken from Table I (Nos 6 and 7)

Thus, it will be seen that in this series of 86 cases of cirrhosis of the liver 64 cases (74.4 per cent) showed all the evidences of 'decompensated portal cirrhosis'. The observations in the present paper are based on a detailed study of these 64 cases.

It may be noted here that no case of cirrhosis of the liver described under the group 'Progressive lenticular degeneration' (Wilson 1912) or 'hepato-lenticular degeneration' (Hall, 1921) was met with in this series.

(3) *Ætiology*

The age, sex and occupational incidence of the 64 cases of 'decompensated portal cirrhosis' studied in this paper are given in the following tables (Tables III, IV and V) —

TABLE III

Showing the age distribution of the 64 cases

Serial number	Age (in years)	Number of cases
1	Below 5	5
2	5-10	1
3	10-15	3
4	15-20	4
5	20-30	10
6	30-40	21
7	40-50	11
8	50-60	9
9	Above 60	

TABLE VII

Showing the distribution according to status of the 64 cases

Serial number	Status	NUMBER OF CASES	
		Adults	Children
1	Poor class	36	3
2	Lower middle class	18	1
3	Upper middle class	4	2
4	Rich class		

Out of the 64 cases 43 were from Vizagapatam district, while 11 were from Godavari, 9 from Ganjam and 1 from Kurnool districts. The majority of the patients admitted into the King George Hospital, Vizagapatam, belong to the Vizagapatam district and this fact explains the apparently greater number of cases seen in this district, in the present series.

It will thus be seen that 'decompensated portal cirrhosis' is more common among the poor and lower middle class people of the Hindu community, between the ages of 20 and 50 years, males are more affected than females, and it is more common among the ryots (cultivators) and other people whose work lies mainly in the fields, though it is also seen in people with other occupations. No case of portal cirrhosis was seen among Europeans or Anglo-Indians.

(4) *Previous history*

The important data noted in the previous history of the 64 cases in the present series are given in Table VIII. It may be seen from the table that 30 cases (46.9 per cent) gave a history of fever with chills for long periods before the onset of the disease and the patients in most of these cases noticed enlargement of the spleen, even before the onset of ascites. It may be pointed out here that malaria is very common in this district. In a few cases history of an irregular fever was present before the onset of the ascites. The high incidence of malaria in this district and the history of fever with chills, suggest that the fever in most of these cases was probably malarial in nature, but, whether it is an integral part of the disease under consideration or not, it is not easy to decide.

Few patients in this series gave a history of dysentery or gastro-enteritis, though the incidence of intestinal diseases in this district is very high. In both the sexes tobacco-smoking is very common in the poor and lower middle class people.

History of hæmatemesis was present in three cases. In two cases it was repeated during a short interval and one patient (case No. 151) succumbed in the hospital to a fatal hæmatemesis. In one case there was a history of repeated attacks of epistaxis for two years prior to admission into the hospital for 'decompensated portal cirrhosis' of three months' duration.

TABLE VIII

Showing the important data noted in the previous history of the 64 cases

Serial number	History of	NUMBER OF CASES	
		Adults	Children
1	Syphilis	3	
2	Anti syphilitic treatment	1	
3	Tuberculosis		
4	Dysentery	15	
5	Gastro enteritis	4	3
6	Fever	5	2
7	Definite malaria	30	
8	Alcohol (a) Arrack	5	
	(b) Toddy	9	
	(c) Brandy	1	
	(d) Other alcoholic drinks		
9	Tobacco smoking	41	
10	Hæmatemesis	3	
11	Epistaxis	1	
12	Bleeding from the gums	1	
13	Jaundice	7	1

It may be seen from Table VIII that the number of cases with a positive history of consumption of alcohol (in any form) is surprisingly low. This is undoubtedly due to the poverty of the poor and lower middle classes who cannot afford to buy alcohol, even if they wish to indulge in it. All the patients in this series were carefully and delicately questioned to elicit the history of alcohol of one variety or another. Only 9 patients gave a history of the drinking of toddy, 5 of arrack and 1 of brandy. Even in these patients only 2 gave a history of excessive drinking, while the others were not habitually addicted to it, being accustomed to drink in small quantities on festive occasions.

The notes of the two cases in which there was a history of excessive drinking (of toddy, arrack or brandy) are given below —

Case No 96 —An aged barber (Hindu) sought admission into the King George Hospital for pain in the right hypochondriac region, of 6 days' duration. He contracted syphilis in Rangoon and had dysentery (for which he was treated at Rangoon) six months ago. He was habituated for the last 20 years to drink toddy daily in large quantities till he was tipsy, and arrack once a week.

All the signs of portal obstruction were present, the left lobe of the liver was tender on palpation, the right being not palpable. The spleen was not enlarged. The patient was empirically given seven emetine injections, though there was nothing abnormal in the microscopic examination of the motion, with no improvement in his condition. He gradually became worse and died in a cholæmic state. A post mortem was not permitted.

Case No 108 —A Hindu, middle aged man, who recently returned from Singapore, where he was employed as a cooly in a mill, was admitted into the King George Hospital for swelling of the abdomen and breathlessness of six months' duration. The patient contracted syphilis at Singapore and was a smoker. He was addicted for the last 14 years to toddy and arrack in moderate amounts on alternate days and large doses of brandy once in every 10 days.

The patient showed all the signs of portal obstruction. The right lobe of the liver was not palpable, while the left lobe (after paracentesis of the abdomen) was found to be hard and finely granular. The spleen was markedly enlarged reaching a level of about 2 inches above the Poupart's ligament.

The liver function was markedly impaired as was shown by the high retention of Rose Bengal (98.6 per cent at the end of the 8th minute) and the diminished tolerance for lactulose. As in the blood and the ascitic fluid Wassermann reactions were positive, the patient was treated with iodides and salyrgan. Paracentesis of the abdomen was done thrice during his stay of 3 weeks in the hospital, and the ascitic fluid was found to be a transudate.

The patient gradually became worse, was unconscious towards the end and died in a state of coma. Post mortem examination showed a multi-lobular cirrhosis of the liver and a marked passive congestion of the spleen.

(5) *Family histories*

There is nothing in the family histories to suggest that the disease is familial, though in two cases there was a history of another member (brother in one case and wife in the other) of the same family having been similarly affected. In two children, in which the disease was the hepatic manifestation of late congenital syphilis, a history pointing to similar affection in the other members of the same family was obtained.

(6) *Dietetic studies*

The diets of all these patients in the present series were carefully investigated with a view to find out if there are any dietetic errors or irregularities to account for the disease. The diets of these patients have been found to be in no way different from the normal people belonging to the same class and caste. The following table

(Table IX) shows that amongst 64 cases there were 58 non-vegetarians and 6 vegetarians —

TABLE IX
Showing the nature of diet in the 64 cases

Nature of diet	NUMBER OF CASES	
	Adults	Children
(1) Non-vegetarians	58	3
(2) Vegetarians	5	3

Though most of the patients were non-vegetarians, meat, mutton or fish, were only taken occasionally once in a week or a fortnight. The diets of most of these patients were deficient in proteins, fats and vitamins, especially A, C and D. The diets of the poor and lower middle class people are very poor, both in quality and quantity on account of their poverty. In most of them the morning meal consisted of a small quantity of cooked rice with 'conjee' and chutney containing excess of chillies, the afternoon meal, of porridge made of 'ragi', 'gantı' or 'cholam' (with water and salt), and the night meal consisted of cooked rice or other cereals ('gantı' or 'ragi'), with soup and chutney, and occasionally cooked vegetables, or buttermilk. Rice was the staple article of diet in most of the cases. Fresh vegetables, dhall, milk or milk products were taken only sparingly and that too occasionally.

There was an excess of chillies, though not of the other condiments, in the dietary of all the patients belonging to the poor and lower middle classes.

The diets of the upper middle class patients were less unsatisfactory and consisted of two principal meals of cooked rice with vegetables, dhall, soup, ghee and buttermilk. Though milk was not often taken by them as such, curds were often taken with the food. Deficiency of proteins and excess of chillies and other condiments were also found in the diet of these patients.

(7) Onset of the disease

The majority of the patients were unable to give the exact time of onset of the disease. As the onset was insidious they had not paid any particular attention to the earlier symptoms of the disease, which should have been vague. Distension of the abdomen was the first symptom to be noticed to warn the patient that something was wrong. A few patients gave a history of discomfort and occasional pain in the upper part of the abdomen, accompanied by dyspepsia and constipation, diarrhoea or some other gastro-intestinal disturbances. They were unable to state whether the fever from which they had suffered before the onset of the disease, was due to malaria or some other cause.

The onset of ascites was acute in 3 cases and slow and gradual in 61 cases, as seen from Table X. In all the 3 cases in which the onset of ascites was rapid, the

patients complained of pain in the epigastric and right hypochondriac regions. The duration of the ascites in the present series of cases, prior to their admission into the hospital, is also given in Table X —

TABLE X

Showing the mode of onset and the duration of the ascites in the 64 cases

Mode of onset and duration of ascites	NUMBER OF CASES	
	Adults	Children
(a) <i>Acute</i> —		
(1) One week	1	
(2) Two weeks	1	
(3) Three „	1	
(b) <i>Slow and gradual</i> —		
(1) 1 month	6	2
(2) 2 months	19	1
(3) 3 „	10	1
(4) 4 „	3	1
(5) 5 „	4	
(6) 6 „	4	
(7) 7 „		
(8) 8 „	1	
(9) 9 „	2	
(10) 10 „	2	
(11) 11 „		
(12) 1 year	3	1
(13) 2 years		
(14) 3 „		
(15) 4 „	1	

It will be seen from the above table that, in most of the cases, the duration of ascites before the patient sought admission into hospital was from 1 to 3 months. In the others, it varied from a few months to one year and only one patient gave the duration of ascites as 4 years.

(8) Signs and symptoms

It is unnecessary to describe here in detail the well-known signs and symptoms of 'decompensated portal cirrhosis'. Some of the important findings on physical examination and the symptoms in the present series of cases are shown in Tables XI and XII —

TABLE XI

Showing the important findings on physical examination in the 64 cases

Serial number	Physical findings	NUMBER OF CASES	
		Adults	Children
1	Fever	4	1
2	Anemia	37	
3	Jaundice —		
	(a) Sub icteric tint of the conjunctiva	28	3
	(b) 'Overt jaundice'	6	
4	Malnutrition	44	
5	Edema —		
	(a) marked œdema of the feet	50	6
	(b) œdema of the abdominal wall	4	1
	(c) general anasarca	4	
6	Dry skin	50	
7	Veins of the anterior abdominal wall —		
	(a) very prominent	32	6
	(b) prominent	18	
	(c) faintly seen	7	
	(d) 'Caput Medusæ'	1	
8	Bleeding piles		
9	Liver —		
	(a) Enlarged	2	4
	(b) Left lobe only—enlarged and hard	4	
	(c) Left lobe only—not enlarged but hard	12	1
10	Spleen —		
	(a) Slight enlargement	16	2
	(b) Moderate "	14	3
	(c) Marked "	8	
11	Ascites	58	6
12	Hernia —		
	(a) Inguinal	1	
	(b) Epigastric	1	
	(c) Umbilical	1	

TABLE XII

Showing the main symptoms in the 64 cases

Serial number	Main symptoms	NUMBER OF CASES	
		Adults	Children
1	Breathlessness	52	6
2	Distension of the abdomen	58	6
3	Emaciation	44	
4	Jaundice	6	
5	Hæmatemesis	3	
6	Bleeding piles		
7	Malena		
8	Diarrhoea	5	1
9	Constipation	53	5
10	General weakness	42	
11	Severe abdominal pain	3	
12	Abdominal discomfort	46	6

It will thus be seen from Table XII that all the patients sought admission into the hospital for *distension of the abdomen*. *Breathlessness*, *abdominal discomfort*, *constipation* and *general weakness* were also troublesome features in most of them, after the onset of ascites. Most of them also observed *gradual loss of weight* and *progressive emaciation*.

Table XI shows that *anæmia*, *malnutrition* (with 'hepatic facies') and *œdema of the feet* were marked features in most of the cases. In 6 cases there was 'overt jaundice', while in 31 cases only a *sub-icteric tint* of the conjunctiva. The *skin* was dry, inelastic and unhealthy in 50 cases and in most of them impetigo was present. The *veins of the anterior abdominal wall* were well marked and prominent in most of the patients. Typical '*caput medusæ*' was present in one. Ascites was a constant feature in all, in most of them, the umbilicus was flattened out or everted due to the pressure of the fluid in the abdomen. Out of the 64 patients, 4 were tapped once, and 6 twice, prior to admission into the hospital for treatment.

The *liver* was enlarged only in two adult patients. In one of them (case No 106), the cirrhotic liver and the ascites were noticed at an exploratory laparotomy in the Gynæcological Wards of the hospital, where an omentopexy was performed, and the patient was later referred to the Medical Wards for further treatment. Only the left lobe of the liver was enlarged and hard in 4 cases.

while in 13, it was not enlarged but definitely hard. In most of the cases, the liver was not palpable.

The spleen was enlarged in most of the cases (43 in this series). It was smooth on the surface and fairly hard with rounded edges.

Hernia which was 'commonly observed' in cases of 'decompensated portal cirrhosis' (47 in a series of 112 cases) by Chapman, Snell and Rowntree (1931), was found only in 3 in this series.

Oral sepsis and fetor of the breath were common features in most of the patients, in whom the teeth were dirty and pyorrhœa alveolaris was present.

Cardiac and respiratory embarrassment was present, and the heart was displaced upwards in a large percentage of patients, due to the pressure of the large quantity of fluid in the abdomen. Organic disease of the heart was not seen in any. In 18 patients (28.1 per cent) the heart was slightly dilated, its sounds were feeble, and soft systolic murmurs were heard in the mitral and pulmonary areas, the second sound of the heart was accentuated in the pulmonary area and faintly heard in the aortic area. The blood-pressure was low in most of the patients.

Apart from the signs and symptoms in the lungs due to the pressure of the fluid in the abdomen, hydrothorax was found in one case, emphysema in another and signs of tuberculosis (which was not active) in 18 cases.

Delirium tremens, muscular tremor and peripheral neuritis—nervous manifestations, commonly associated with alcoholic excess, in cases of cirrhosis of the liver (Rolleston and McNee, 1929a)—were not seen in any of the patients in this series. Vague symptoms of tingling or numbness in the extremities were present in some, in whom the deep reflexes were lost. Though the skin was unhealthy in most of them, severe pruritus was present only in 6.

(9) *Diagnosis and differential diagnosis*

As already mentioned, the symptoms of portal obstruction were definite in all the patients selected for this study and the diagnosis was further verified after a detailed investigation. In most of them the difficulty was not in the diagnosis of portal obstruction but in finding out the cause of cirrhosis.

It is not necessary to discuss the differential diagnosis of 'decompensated portal cirrhosis' in detail, as the several conditions mentioned (*cf.* Rolleston and McNee, 1929b) in the differential diagnosis of the disease do not in any way differ in this country.

(10) *Duration and course of the disease*

Unfortunately, in all these cases it was not possible to follow them up after discharge from the hospital. On account of their limited stay in the hospital, and in the absence of follow-up records, it is difficult to gauge exactly the duration and course of the disease.

The average duration of stay of the patient in the hospital was less than three weeks. Only 14 were in the hospital for longer periods, varying from 1 to 5 months. The disease ran an apyrexial course in most of the patients during their stay in the hospital but 4 patients had a low, irregular and intermittent type of

fever Paracentesis was done in the large majority of patients to afford relief from the pressure effects of the fluid in the abdomen. Many patients became progressively worse and 14 of them died in the hospital.

The duration of life after the onset of ascites in the 14 fatal cases is given below —

Duration of life after the onset of ascites	Number of cases
Less than one month	1
2 months	3
3 „	4
6 „	1
7 „	3
12 „	2

In some of these patients (who died), there was a terminal rise of temperature, while in others, the temperature was sub-normal towards the end. A few patients showed a rise of temperature on the day of the paracentesis.

(11) *Prognosis and mortality*

Though 'the prognosis in cases of cirrhosis of the liver associated with syphilis has been considered to be better than in other types of cirrhosis' (Chapman *et al*, 1931), most of the patients in the present series who showed either clinical or serologic evidences of syphilis did not respond well to anti-syphilitic treatment, as the disease was much advanced by the time they sought admission into the hospital. The following case illustrates the beneficial effects of anti-syphilitic treatment, in cases of cirrhosis of the liver associated with syphilis, if it is begun early and persisted in for a long time —

Case No. 1 — A Hindu boy, aged 16 years, who was admitted into the King George Hospital, Vizagapatam, for swelling of the abdomen of 8 months' duration, showed all the signs and symptoms of portal obstruction. The right lobe of the liver was not palpable, but the left lobe was enlarged to five finger-breadths below the xiphisternum in the middle line and hard. The spleen was enlarged to the level of the umbilicus and hard.

Though there were no clinical evidences of syphilis, the Wassermann reactions of the blood and the ascitic fluid were positive and therefore the patient was given a complete course of anti-syphilitic treatment (iodides by mouth and intramuscular injections of mercury cream). The patient stayed in the hospital for about 4½ months, during which period he was tapped 5 times. The patient gradually improved and the abdomen filled up with fluid less rapidly than before. At the time of discharge, there was only a small quantity of free fluid in the abdomen and the general condition of the patient was good. The patient was seen twice at intervals of two months after discharge from the hospital and on both occasions there was no fluid in the abdomen, and the liver and spleen showed diminution in size.

The prognosis in most of these patients was bad on account of the advanced stage of the disease at the time of admission into hospital, and the incomplete treatment due to the brief period of stay in the hospital.

Fig 1a

Fig 1b

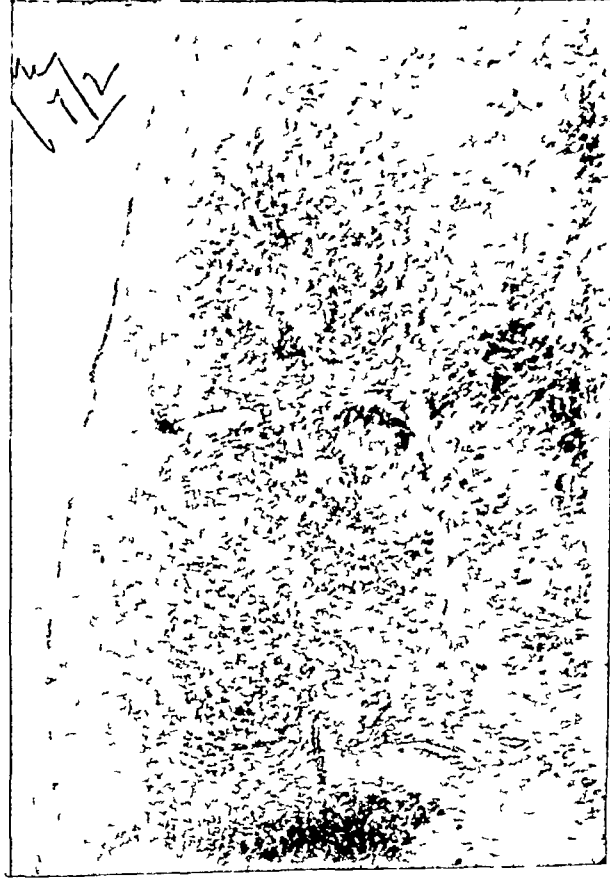


Fig 2

Fig 1a (case No 138) Photograph of the surface of the liver showing 'nodular hyperplasia' Weight of the organ 35 oz
Fig 1b (case No 138) Photograph of the section of the liver

Fig 2 (case No 138) Low power photomicrograph of the liver showing well marked thickening of the capsule with puckering of the surface and marked increase of the interlobular connective tissue The normal structure of the liver lobules is completely lost
(For detailed description see text)

There were 14 deaths (21.9 per cent) in the hospital in this series. Three patients were discharged moribund. Though in a few there was temporary relief, most of them showed no improvement at the time of discharge from hospital.

(12) Mode of termination

The modes of death in the 14 fatal cases are as follows —

Coma	10
Heart failure	1
Delirium, mania and terminal coma	1
Coma and terminal delirium	1
Hæmatemesis	1

Ten patients in this series gradually became comatose and died, without hæmorrhage or any infection. The type of coma is peculiar and as remarked by Rowntree (1930) 'it is the appearance of normal sleep that is so impressive'. One patient (case No. 33) became delirious and acutely maniacal after paracentesis of the abdomen and he remained in that condition for 2 days, after which he became deeply comatose and died. Post-mortem examination showed multi-lobular cirrhosis of the liver. Another patient (case No. 151), who was apparently improving, suddenly died after a profuse hæmatemesis. Post-mortem examination showed a coarsely nodular cirrhosis of the liver and dilated and tortuous veins (submucous) in the cardiac end of the œsophagus.

(13) Post-mortem appearances

Of the 12 cases in which a post-mortem examination was performed, the liver was finely granular in 4 and coarsely nodular in 8. In a child (case No. 162), in which the liver was finely granular and the left lobe markedly contracted, suggesting phylloidal cirrhosis, microscopical examination showed typical multi-lobular cirrhosis. Macroscopical appearances of the liver in the other cases (3 in this series) were of the finely granular variety and microscopically all of them were of the multi-lobular cirrhosis type. In one instance there was marked perihepatitis and polyserositis.

The macroscopic appearances of the liver in the other cases were those of the coarsely nodular type and microscopically the liver showed appearances typical of multi-lobular cirrhosis. The following is an illustrative case —

Case No. 138 — (See Plate XX) A Hindu middle aged woman, who was admitted into the King George Hospital, for swelling of the abdomen and jaundice of two months' duration, died in the hospital in a cholæmic state.

The Wassermann reactions of the blood and the ascitic fluid were positive, there was marked retention of the Rose Bengal dye at the end of the 8th minute, and diminished tolerance for lævulose. The liver was not palpable but only the spleen was enlarged to a finger breadth below the costal margin and hard.

On post-mortem examination, the liver was found to be shrunken and much diminished in size. It weighed 35 oz. The capsule was thickened and dense, grey bands of thickened capsule were seen, especially in the depressions between the nodules. Somewhat uniformly granular appearance was seen only over a small area on the diaphragmatic surface of the upper lobe. The rest of the lobes presented only a coarsely nodular appearance. The organ was very resistant to the cutting knife and showed on section greenish liver lobules of varying size separated by thick grey bands of fibrous tissue. This was more marked in the right lobe. The consistency of the organ was very firm.

The microscopic examination of the liver showed well marked thickening of the capsule with puckering of the surface. There was marked round cell infiltration in the Glisson's capsule and interlobular connective tissue. The distribution of the fibrous tissue was typical of multi lobular cirrhosis.

The spleen weighed 16 oz. and showed typical appearances of passive venous congestion. The bile staining of the liver in this case was evidently a terminal condition.

In the 12 cases, the spleen was enlarged markedly in 5, moderately in 4, and slightly in 3, in one case there were miliary tubercles on the peritoneum, being part of a terminal generalized miliary tuberculosis. Marked collateral circulation was found in 5 patients. In one case (No 166) the whole of the omentum was drawn to the left side of the abdomen, where it was firmly adherent to the abdominal wall below the level of the spleen forming well-marked collateral circulation. No evidences of dysentery, recent or old, were found in the intestines in any of the fatal cases, only terminal colitis was observed in one case.

(14) *Laboratory findings*

(a) *Hæmatology* — In most of the patients, the blood-picture was that of a secondary anæmia, with a low colour index. Though clinically anæmia was found only in 37 patients, in most of them the hæmoglobin percentage was lower than normal, and in 26 it was very low. In case No 1 the total red-cell count was 1.6 millions per c mm. and the hæmoglobin 35 per cent.

In the majority of the cases, the total red-cell and white-cell counts were below normal. Slight leucocytosis was present in 5 cases.

The differential counts were normal, except that there was a slight predominance of lymphocytes in a small number of cases.

The hæmatological findings in the following case (in which diagnosis of multi-lobular cirrhosis was verified post-mortem) are very nearly typical of those seen in the majority of patients —

Case No 42 —

Hæmoglobin	45 per cent
Total red cell count	2.4 millions per c mm
Total white cell count	2,688 per c mm
<i>Differential count</i>	
Polymorphonuclear neutrophils	78 per cent
Lymphocytes	19 „
Eosinophils	5 „
Monocytes	3 „
Mast cells	1 „

(b) *The fragility of the red-blood cells* was diminished in most of the cases, while in the rest it was within normal limits. Increased fragility of the red cells was not found in any.

The *coagulation time* of the blood was either normal or slightly delayed.

(c) *The van den Bergh reaction* — Though 'overt jaundice' was only seen in 6 patients, a direct positive reaction (delayed) or an indirect reaction was obtained in most of them. The bilirubin content of the serum in these was slightly higher than the normal limits but the jaundice was 'latent'.

(d) *Scrological tests*—In the few cases in which the 'aldehyde test' of Napier (1921, 1922) was done, it was found negative

The results of the *agglutination tests with the dysentery group of organisms* show that positive results were obtained in a great number of patients who did not give a history of dysentery. A positive reaction to *B dysenteriae* (Flexner) only was obtained except in a few patients who gave a positive reaction to both *B dysenteriae* (Flexner) and *B dysenteriae* (Shiga)

The results of the Wassermann reaction of the blood in the 55 patients on whom it was done are shown below —

(++) Strongly positive	28
(+) Positive	13
(±) Doubtful	4
(-) Negative	10

It will be seen that the Wassermann reaction of the blood was positive in the majority of patients

The results of the examination of the blood are summarized in the following table —

TABLE XIII

Showing the analysis of the results of the examination of the blood in the 64 cases

Serial number	Nature of examination	Number of cases (percentage)
1	Percentage of hæmoglobin —	
	(a) Normal	12.5
	(b) Less than normal	46.9
	(c) Very low	40.6
2	Total red cell count —	
	(a) Normal	33.4
	(b) Less than normal	66.6
3	Total white cell count —	
	(a) Normal	50.5
	(b) Less than normal	61.8
	(c) Slight leucocytosis	7.7

TABLE XIII—*concl'd*

Serial number	Nature of examination	Number of cases (percentage)
4	Fragility of the red cells —	
	(a) Normal	19.0
	(b) Diminished	91.0
	(c) Increased	
5	Blood coagulation time —	
	(a) Normal	41.0
	(b) Slightly delayed	59.0
6	van den Bergh reaction —	
	(a) Direct —	
	(i) Immediate	8.2
	(ii) Biphasic	12.3
	(iii) Delayed	61.0
	(b) Indirect	11.0
	(c) Negative	7.5
7	Agglutination tests with dysentery organisms —	
	(a) Positive	55.8
	(b) Negative	44.2
8	Wassermann reaction —	
	(a) Strongly positive	50.9
	(b) Positive	23.6
	(c) Doubtful	7.3
	(d) Negative	18.2

(c) *Liver function tests* — The liver function was diminished in most of the cases as shown by the retention of the Rose Bengal at the end of the eighth minute, the greater number of positive *lævulose-tolerance tests* (diminished tolerance for lævulose), and the leucopenia in the *Widal's hæmolytic crisis*. The value of these tests and

then interpretation, especially in cirrhosis of the liver, have been discussed in previous papers (Radhakrishna Rao, 1932, 1933*a*, 1933*b*)

(f) *Biochemical findings of the blood*—The constant features found in most cases of 'decompensated portal cirrhosis' were the low fasting blood-sugar and the low cholesterol content of the plasma. The urea content of the blood was variable, while the creatinin and non-protein nitrogen were either within or slightly above the normal limits. 'The chemistry of the blood in cirrhosis of the liver' is discussed in detail in a separate paper (Radhakrishna Rao, 1933*c*)

(g) *Urine*—There was a trace of albumin in the urine of some of the patients, but it disappeared after paracentesis of the abdomen. In all of them the urine was scanty and high coloured. Bile salts and bile pigments were present in the urine in those with 'overt jaundice', and urobilin or urobilinogen in nearly all of them. In some instances the urine gave a strong reaction for indican. Glycosuria was not present in any.

The renal efficiency, as shown by the urea concentration test, was fairly good in most of the patients.

(h) *Motions*—Microscopic examination of the faeces showed hookworm infection in nearly all patients.

(i) *Ascitic fluid*—The ascitic fluid was a transudate in all the cases (48 in this series) in which paracentesis of the abdomen was performed. The specific gravity of the fluid varied between 1004 and 1012. The total protein content was less than 1.5 per cent. The fluid was clear, straw-coloured or greenish-yellow (slightly bile stained in 6 cases) and faintly alkaline in reaction. It did not coagulate either spontaneously or on standing. The centrifuged deposit contained endothelial cells and in most of the cases lymphocytes (though there was no evidence of tuberculous peritonitis).

The culture of the fluid was sterile and in few cases staphylococci were grown, probably due to contamination. The organism isolated from the ascitic fluid in one case (No. 2) did not conform to any of the biological tests for the known pathogens.

The results of the intraperitoneal inoculation of the ascitic fluid into guinea-pigs were negative in the few cases in which it was done.

The Wassermann reactions of the ascitic fluid were similar to those obtained by the blood-serum in most of the cases, but the results of the agglutination tests with dysentery organisms did not compare favourably with those of the blood-serum, either a negative result or a lower titre being obtained in many instances. The value of the ascitic fluid in the Wassermann reaction and the agglutination tests (with dysenteric organisms) is discussed in a separate paper (Radhakrishna Rao, 1933*d*).

(15) *Treatment*

The results of treatment of these patients were very unsatisfactory. On account of the advanced condition of the disease at the time of admission into hospital, and the short duration of treatment, the response to it was very poor.

practice at the Johns Hopkins Hospital there was no case of advanced cirrhosis due to malaria'

Thus, all the above evidences confirm that malaria cannot be considered to be a causal factor in the production of cirrhosis of the liver, though it may be an important predisposing cause

The rôle of *dysentery* in the causation of ascites and cirrhosis was discussed in detail in a previous paper (Radhakrishna Rao, 1932). There was no history of chronic dysentery to suggest that it might have been an important factor in the causation of 'decompensated portal cirrhosis'. Hameed (1933) investigated the so-called 'endemic ascites' in the King George Hospital, Lucknow, and showed that the causation of ascites in these cases is not due to 'chronic superior peritonitis' (Sprawson) or 'chronic dysenteric peritonitis' (Megaw) but to cirrhosis of the liver

On account of the marked enlargement of the spleen in some of the cases (8 cases in this series), the question of *primary splenic anaemia with terminal cirrhosis* (so-called Banti's disease) in the causation of 'decompensated portal cirrhosis' requires consideration. As all these patients were seen only after the onset of ascites, it was difficult clinically to differentiate cases of primary portal cirrhosis from those of the so-called Banti's disease, as the clinical picture is almost the same in the later stages. But, out of the 12 cases (in this series) in which an autopsy was performed, the microscopical appearances of the spleen in 3 cases were typically those of 'splenic anaemia'. Unless these cases are followed from the beginning, a clinical diagnosis of 'splenic anaemia' ending in cirrhosis may not be possible. Thus, 'primary splenic anaemia' was the cause of 'decompensated portal cirrhosis' in at least 3 cases (4.7 per cent). In a previous paper (Trinmurti and Radhakrishna Rao, 1933) we have shown from the study of the spleens removed at autopsy from cases of cirrhosis of the liver, that splenic anaemia ending in cirrhosis of the liver was seen in 10.1 per cent of cases. In these cases, the enlargement of the spleen was much bigger than that seen in cases of mere passive congestion due to portal obstruction. De (1932) reported cases of splenomegaly terminating in cirrhosis of the liver and ascites from Bengal.

The rôle of *alcohol* in the production of 'decompensated portal cirrhosis' does not require extensive consideration. In the majority of patients there was no history of excessive drinking of alcohol in any form, sufficient to produce any effect on the liver. Even in the two persons (Nos 96 and 107) in whom there was a history of excessive drinking of toddy, arrack or brandy, other stronger evidences were present to account for the causation of cirrhosis. In fact one of these patients (case No 107) was treated for syphilitic cirrhosis, during his stay in the hospital. It may be remarked here that cases of portal cirrhosis were not seen in the scavengers of this town, who are rather heavy drinkers of toddy or arrack. The extensive statistics of Rowntree (1927) clearly showed the probable relationship between alcoholism and cirrhosis of the liver. But, in the present series of cases alcohol cannot be considered as an important factor in the causation of portal cirrhosis.

All the six children in this series showed definite clinical evidences of *congenital syphilis* and the Wassermann reactions of the blood and ascitic fluid were positive. In one case (No 162) in which an autopsy was performed, the liver showed a finely granular appearance and on microscopical examination the distribution of the

fibrous tissue was typically inter-lobular, similar to that seen in multi-lobular cirrhosis. This is an example of multi-lobular cirrhosis occurring in young subjects with congenital syphilis. This is considered as an hepatic manifestation of delayed congenital syphilis by Rolleston and McNee (1929g) who remark —

‘Every now and again the liver of a child with stigmata of congenital syphilis shows ordinary cirrhosis. The arrangement of the two lesions is so dissimilar that inter-cellular cirrhosis cannot be thought to be transformed into multi-lobular cirrhosis, it would rather lead to diffuse fibrosis or gummatous change. It seems probable that the inter-cellular cirrhosis undergoes absorption, but that some vulnerability or diminished resistance of the liver is left behind. If causes that tend to produce ordinary cirrhosis then arise, this change will be readily produced, on the other hand, the multi-lobular cirrhosis may be comparable to general paralysis of the insane, or a lesion due to parenchymatous syphilis’

It is difficult to determine the nature of fibrosis from the clinical findings alone in the other children (5 cases) with evidences of congenital syphilis. Ascites is also common in inter-cellular cirrhosis of the liver due to late congenital syphilis. Counsellor and McIndoe (1926), Judd and Counsellor (1927) and McIndoe (1928) showed the intertwining of the branches of the portal and biliary systems in the liver suggesting the possibility of the strangling effect of one system on the other in hepatic disease. Periductal or pericellular fibrosis can thus easily interfere with the venous flow and result in ascites.

In most patients the diet was deficient in proteins, fats and vitamins and there was excessive consumption of chillies or spices. These findings, together with the history, in a few, of dyspepsia or other gastro-intestinal disturbances before the onset of ascites, suggest the consideration of *dietetic errors* in the ætiology of ‘decompensated portal cirrhosis’. Rolleston and McNee (1928h) pointed out that cases of non-alcoholic cirrhosis of the liver in Indians were reported by Budd (1857), Young (1899) and Sutherland (1905), the causation of cirrhosis of the liver in these cases was explained to be due to dyspepsia and auto-intoxication from the bowel as a result of intestinal fermentation induced by the ingestion of spices, condiments or other stimulating articles of food. Though these dietetic errors are important factors in the causation of portal cirrhosis, it is difficult to determine their exact rôle in the present series of cases. A more detailed investigation into the diets of the people is necessary to find out the exact relationship between dietetic errors and ‘decompensated portal cirrhosis’, and for the present they can be considered only as important predisposing factors.

The rôle of *syphilis* in the development of hepatic cirrhosis requires a detailed consideration on account of the serological evidence of syphilis in 74.5 per cent of cases in the present series. O’Leary, Greene and Rowntree (1929) showed that both biliary and portal types of cirrhosis occur in syphilitic cirrhosis of the liver. They reported cases of portal cirrhosis in which syphilis was the chief ætiologic agent.

Syphilis may possibly produce portal cirrhosis in different ways. The acute destructive hepatitis produced by syphilis, if recovered from, may result in portal cirrhosis. Indirectly syphilis may predispose to any of the causes which ordinarily

tend to produce portal cirrhosis. As pointed out before, the therapeutic use of arsenical preparations in the treatment of syphilis may itself be a potent factor in the causation of portal cirrhosis. It may be pointed out here that it may not be possible clinically, or even pathologically, to differentiate cases of idiopathic portal cirrhosis from those of portal cirrhosis produced by syphilis.

If a positive Wassermann reaction is taken as definite evidence of syphilitic infection, then syphilis may be regarded as an important ætiological factor in the production of 'decompensated portal cirrhosis' in the present series of cases.

Oral sepsis and intestinal intorication were common features in most of the cases. On account of the focal sepsis and the 'nodular hyperplasia' of the liver seen in some of the cases in which an autopsy was performed, the question naturally arises whether the present series of cases can be grouped under 'toxic cirrhosis' (Malloy, 1911). It has been shown that repeated attacks of subacute hepatic necrosis due to some toxic or toxi-infectious processes, result in 'toxic cirrhosis'. Whatever the injurious agent might be it is not sufficient to cause rapid and fatal widespread hepatic necrosis, but sufficient time is allowed for regeneration of liver-cells. Althausen (1931) has shown that the carbohydrate metabolism of the liver remains normal in cases of toxic cirrhosis, though the dye, 'Rose Bengal', excretion is markedly impaired. It has not been possible to utilize these findings in differentiating cases of 'toxic cirrhosis' from those of ordinary portal cirrhosis in the present series of cases as most of them were seen in the terminal stages of the disease, when there is a failure of all the functions. There was a history of jaundice prior to the onset of ascites in 7 cases. In the light of these findings it is worth while making further detailed investigation to determine whether focal infections or other toxic or toxi-infectious processes are responsible for the causation of portal cirrhosis, which is commonly seen in the cultivators belonging to the rural areas of this district. The high incidence of portal cirrhosis in people whose main work lies in the fields also suggests to us to be on the lookout for any toxic or toxi-infectious agent arising from this source. Similar cases of non-alcoholic type of portal cirrhosis commonly seen in cultivators were described from Lucknow (Hameed, 1933), in North China they were termed as 'farmer's cirrhosis' (McIntosh, 1932).

Thus, it will be seen from the above discussion that 'congenital syphilis' was apparently the chief ætiological factor in 6 cases of 'decompensated portal cirrhosis', primary splenic anæmia in 3 cases and alcohol in 2 cases (there was an infection of syphilis as well in these two cases). In the other cases (53 in this series) the exact causation of 'decompensated portal cirrhosis' is far from clear. Malaria, syphilis, dietetic errors and focal sepsis appear to be important ætiological factors. Further investigation is necessary to determine the exact rôle of dietetic errors and focal infections in the causation of 'decompensated portal cirrhosis'.

(b) *Pathogenesis of ascites in portal cirrhosis*

The production of ascites is commonly considered to be due to the portal hypertension consequent on the obstruction to the flow of blood in the cirrhotic liver. On account of the control of ascites, though temporarily, in a few patients in this series by the administration of ammonium chloride and salyrgan, an organic mercurial preparation, it may be remarked that though mechanical obstruction to

the flow of the portal blood and the consequent portal hypertension are important factors in the production of ascites in 'decompensated portal cirrhosis', there are other factors as well which require careful consideration. There is also strong evidence to suggest that hepatic insufficiency itself may be responsible for the production of ascites in some cases.

(c) Treatment

Chapman, Snell and Rowntree (1931) reported very favourably the results of the medical treatment by mercurial diuretics, ammonium salts and a special diet in cases of 'decompensated portal cirrhosis'. In the present series of cases, the response to this line of treatment was very poor on account of the advanced stage of the disease in most of the patients before treatment was started, and the incomplete treatment in the few others who were suitable for this type of medical treatment. Encouraging results might be obtained even in the present type of cases of 'decompensated portal cirrhosis', if the patients are carefully selected for treatment in the early stages of the disease and given a fairly good trial.

It is unnecessary to discuss here at length the injurious effects of vigorous anti-syphilitic treatment in cases in which there is serologic evidence of syphilis. As shown before arsenic itself is an important factor in the production of portal cirrhosis. In the treatment of hepatic syphilis, O'Leary (quoted by Snell and Weir, 1927) recommends the administration of potassium iodide and mercury for several months before mercury is given byunction or injection, in cases which require intensive treatment, arsphenamine may be cautiously used, but only after the prolonged preliminary treatment with iodides and mercury by mouth.

Apparent improvement in the ascites may not always indicate a good prognosis, as the patients may suddenly die of hepatic toxæmia or gastro-intestinal hæmorrhage. In one of the cases (No 151) referred to before, the patient died suddenly after a profuse hæmatemesis, though he was apparently improving so far as the condition of the ascites was concerned. The main cause of death in most of the fatal cases was hepatic toxæmia.

SUMMARY AND CONCLUSIONS

Careful investigation in a series of unselected cases of cirrhosis of the liver in the Medical Wards of the King George Hospital, Vizagapatam, showed that many of the patients presented a typical clinical picture of 'decompensated portal cirrhosis'. These cases were further studied and the results of the investigation into 'decompensated portal cirrhosis' are given in this paper.

It is shown that 'decompensated portal cirrhosis' is more common among the poor and lower middle class people of the Hindu community, between the ages of 20 and 50 years, males are more affected than females, and the disease is more common among the ryots (cultivators) and other people whose work lies mainly in the fields, though people with other occupations are not exempt.

Most of the patients were not addicted to alcohol in any form. A history of indulgence in toddy, arrack or brandy was obtained only in a very few cases. The

diets of these patients were carefully investigated and found to be deficient in proteins, fats and vitamins, especially A, C and D, and to have an excess of chillies or other condiments

The onset of the disease and the important signs and symptoms and the pathological features in a few fatal cases are described. The post-mortem appearances in most cases were those of multi-lobular cirrhosis, with 'nodular hyperplasia'

It is a progressive and rapidly fatal disease, most of the patients dying within one year after the onset of ascites, in a comatose state (cholæmia) due to hepatic toxæmia. Death from gastro-intestinal hæmorrhage (seen only in one case) does not appear to be the common mode of termination.

The hæmatological, bacteriological and biochemical findings and the results of inoculation of the ascitic fluid into guinea-pigs have been noted for those cases in which these investigations were carried out. Tests for liver function were done in all the patients.

It is pointed out that the treatment of these patients was very unsatisfactory owing to the advanced stage of the disease by the time they sought admission into hospital.

The ætiology of the disease is discussed in detail and it is shown that the 'ascitic phase of cirrhosis is not necessarily the end stage of a specific process but that it may be due to a variety of diffuse hepatic lesions'. Congenital syphilis and primary splenic anæmia were shown to be the chief ætiological factors in a few cases. The exact causation in the rest is far from clear, but it is pointed out that malaria, syphilis, dietetic errors and focal infections appear to be important ætiological factors. It is suggested that further investigation into the exact rôle of dietetic errors and focal infections may prove that the main type of the disease is probably of the nature of 'toxic cirrhosis' (Mallory).

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STATEMENT A

Showing the incidence of cirrhosis of the liver, as gleaned from the statistics of the King George Hospital, Vizagapatam

	1931-1932 (JULY 1931 TO APRIL 1932)		1932-1933 (JULY 1932 TO APRIL 1933)		Total in patients	Total cases of cirrhosis of the liver	Total percentage
	Total number of cases admitted	Number of cases of cirrhosis of the liver	Total number of cases admitted	Number of cases of cirrhosis of the liver			
(a) In patients (Medical Wards) —							
(1) Males	1,522	41	1,107	32	2,629	73	
(2) Females	372	5	250	7	622	12	
(3) Children	165	12	104	2	269	14	
Totals					3,520	99	2 81
(b) Total out patients (All departments)	120,591		105,363				

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NOTES ON SOME INDIAN SPECIES OF THE GENUS *PHLEBOTOMUS*

Part XXXVI.

DIAGNOSTIC TABLE FOR THE MALES OF THE SPECIES RECORDED FROM INDIA

BY

LIEUT -COLONEL J A SINTON, M D , D SC , I M S

(*From the Malaria Survey of India, Kasauli*)

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SINTON (1924a) published a provisional diagnostic table to include the males of the ten species of *Phlebotomus* recorded at that time from India. Since then the number of recorded species has risen to 33, and although Sinton (1932a, 1933d) has published tables to include the females of these species, no table for the males has appeared.

As in the identification of the females, it has been found convenient to divide the species into two main groups --

(a) those with erect hairs on the dorsal aspect of some of the abdominal segments from II to VI and (b) those in which no erect hairs are present on these segments. In the species with scanty erect hairs on these segments (sub-genus *Sintonius* of Nitzulescu, 1931), these hairs are more scanty on the males than on the females in most specimens. For this reason several of these species have been included into two places in the table.

On account of the ease with which the hairs on the abdomen may be disarranged in capture and subsequent manipulation, it is often difficult to be certain of the arrangement of the hairs on the dorsum of the abdomen of fresh and dry specimens. In specimens which have been mounted, and more especially if stained (Sinton, 1925d), it is comparatively easy to determine the original disposition of the hairs from a study of the nature of the scars left on the abdominal segments (Plate XXI, fig 6) (Sinton, 1932a, Plate III, fig 1).

In Appendix I has been given a short explanation of many of the terms used in the table. In Appendix II reference has been given to the more important papers which have appeared in recent years on the morphology of the species of *Phlebotomus*.

recorded from India In case of doubtful identification workers are referred to the more complete descriptions given in the literature listed

DIAGNOSTIC TABLE FOR THE MALES OF THE INDIAN SPECIES OF THE
GENUS *PHLEBOTOMUS*

1	Species with erect hairs on the dorsal aspect of some of the abdominal segments from II to VI	2
	Species with no erect hairs on the dorsal aspect of any of the abdominal segments from II to VI	16
2	Species with numerous erect hairs on abdominal segments II to VI, usually forming tufts at the posterior ends of the segments, genitalia with characteristic morphology (Plate XXI, figs 1-4, 9-11, 14, 17, 18)	3
	Species with scanty erect hairs on some of the abdominal segments from II to VI, seldom forming tufts (Plate XXI, fig 6), genitalia of the 'minutus' type (Plate XXII, figs 20, 24, 26, 29, 30)	12
3	With only 4 well developed spines on the distal segment of the superior clasper (Plate XXI, figs 1-4)	4
	With 5 well developed spines on the distal segment of the superior clasper (Plate XXI, figs 9-11, 14, 17, 18)	7
4	With intermediate appendage markedly lobed no pedunculated brush on the inner surface of proximal segment of superior clasper (Plate XXI, figs 1, 2)	5
	With intermediate appendage simple, pedunculated brush present on inner surface of proximal lobe of superior clasper (Plate XXI, figs 3, 5)	6
5	With 1 apical, 2 median and 1 proximal spines on distal segment of superior clasper, intermediate appendage bilobed, pointed triangular intromittent organ with no lateral spines (Plate XXI, fig 1)	P. colabaensis.
	With 2 apical or sub apical and 2 proximal spines on distal segment of superior clasper, intermediate appendage trilobed, intromittent organ slender with spine along each side (Plate XXI fig 2)	P. maynei.
6	With 2 spines on distal segment of superior clasper apical, carried on two approximately equal tubercles (Plate XXI, fig 3), wing large (Plate XXI, fig 8), tip of IIIrd antennal segment reaching more than half way to end of proboscis, peduncle of brush on superior clasper long with more curved hairs (Plate XXI, fig 3)	P. sergenti.
	With 1 spine on distal segment of superior clasper apical and other markedly sub apical, carried on very unequal tubercles (Plate XXI, fig 4), wing relatively small (Plate XXI, fig 7), tip of IIIrd antennal segment not reaching half-way to end of proboscis, peduncle of brush on superior clasper short with straighter hairs (Plate XXI, fig 5)	P. sergenti var alexandri.
7	Distal segment of superior clasper very elongated, having parallel sides and 5 short spines, inferior clasper with 2 apical spines, intermediate appendage with 3 characteristic lobes (Plate XXI, fig 9)	P. papatasi.
	Distal segment of superior clasper irregular in shape and with 5 long spines, inferior clasper with no spines (Plate XXI, figs 10, 11, 14, 17, 18)	8

- 8 With 3 spines on distal segment of superior clasper apical or sub apical distal end of intermediate appendage like end of crochet hook, large spine on either side of intromittent organ (Plate XXI, fig 11), halter of characteristic, irregular, flattened shape (Plate XXI, fig 13) **P. newsteadi** 9
- With only 2 spines of distal segment of superior clasper apical or sub apical (Plate XXI, figs 10, 14, 17, 18), halter of usual drum stick shape (Plate XXI, fig 12)
- 9 Tri lobed intermediate appendage, spine on either side of intromittent organ (Plate XXI fig 10), very short 4th palpal segment, cross vein 2-3 markedly distal to end of sub costa (Plate XXI, fig 16) **P. argentipes.**
- Simple intermediate appendage no spine on either side of intromittent organ (Plate XXI, figs 14, 17, 18), 4th palpal segment relatively longer, cross vein 2-3 almost level with end of sub costa (Plate XXI, fig 15) 10
- 10 With pedunculated tuft of hairs on inner side of basal segment of superior clasper (Plate XXI, fig 14) **P. eleanorae.** 11
- With no such pedunculated brush
- 11 Intromittent organ with sub apical tubercle (Plate XXI, fig 18) pompetta lying near middle of abdomen **P chinensis.**
- Intromittent organ with rounded apex and no tubercle (Plate XXI, fig 17), pompetta lying close to hypopygium **P major.**
- 12 Distal end of genital filament markedly expanded (Plate XXII, fig 20) large irregular projection inwards from each side of buccal cavity (Plate XXII, fig 19), no geniculate spines on IIIrd antennal segments, Newstead's spines on both 2nd and 3rd palpal segments, wide scales on thoracic pleura (vide also No 26) **P. squamipleuris.**
- Distal end of genital filament not expanded (Plate XXII, figs 21, 24, 26 30), no large lateral projections into buccal cavity (except in *P hodgsoni*) (Plate XXII, figs 25 27, 28, 31), geniculate spines on IIIrd antennal segments (except in *P cadithi*) no Newstead's spines on 2nd palpal segment, no wide scales on thoracic pleura 13
- 13 Pigmented area large, transverse and oval, single curved row of 30 or more fairly well developed, contiguous buccal teeth (Plate XXII figs 23, 27) 14
- Without large oval transverse pigmented area, buccal teeth less numerous and usually distinctly separated (Plate XXII, figs 25 28 31) 15
- 14 Intermediate appendage with small, distinct spinose, ventral lobe intromittent organ with blunt end (Plate XXII fig 21) buccal teeth about 50 in number (Plate XXII fig 23), non deciduous spine on distal segment of superior clasper more median in position (Plate XXII fig 22), palpal formula 1, 2 4, 3, 5, distal end of IIIrd antennal segment not projecting beyond end of proboscis **P. hodgsoni**

* The scars of the erect abdominal hairs are sometimes absent or difficult to see in the males of this species. It has therefore been shown in two places in the table (vide No 26) Theodor (1931) thinks the African *P squamipleuris* is different from the Indian one, and proposes the name *P squamipleuris* var *indicus* for the latter

- Intermediate appendage without any marked spinose lobe, intromittent organ with pointed end, non deciduous spine on distal segment of superior clasper more distal (Plate XXII, fig 26), palpal formula 1, 2, 3, 4, 5, distal end of IIIrd antennal segment projects beyond end of proboscis
- P. hospitii.**
- 15 Pigmented area very small or absent, row of about 8 widely separated buccal teeth, posterior portion of buccal cavity markedly basin shaped in outline (Plate XXII, fig 31), palpal formula 1, 2, 4, 3, 5
- P. christophersi**
- Pigmented area larger, buccal teeth 20 or more, usually with remnants of an anterior row (Plate XXII, figs 25, 28)
- 16 With row of about 20 evenly spaced, comparatively large, buccal teeth (Plate XXII, fig 28), intermediate appendage almost equal in length to inferior clasper (Plate XXII, fig 29), palpal formula 1, 2, 3, 4, 5, antennal formula I over IV to XV, Newstead's spines about 12 on basal fourth of 3rd palpal segment
- P. eadithae.**
- With row of about 30 small buccal teeth, arranged in groups (Plate XXII, fig 25), intermediate appendage much shorter than inferior clasper (Plate XXII, fig 26), palpal formula 1, 2, 4, 3, 5, antennal formula I over III to XV, Newstead's spines about 30
- P. clydei.**
- 17 With 4 spines on distal segment of superior clasper all distinctly apical or sub apical (Plate XXIII, figs 45, 47, 51, 54, Plate XXIV, figs 58, 61, 64, 67, 68, 70, 73)
- 22
- With only 2 spines distinctly apical or sub apical and 2 median on distal segment of superior clasper (Plate XXII, fig 32, Plate XXIII, figs 37-39, 41, 42)
- 18
- 18 Intromittent organ with broad wedge shaped end (Plate XXII, fig 33), ratio α over β about 2
- P. zeylanicus**
- Intromittent organ with comparatively narrow end (Plate XXIII, fig 36)
- 19
- 19 Small non deciduous spine on distal segment of superior clasper distinctly proximal to all other spines (Plate XXIII, fig 37), palpal formula 1, 2, 3, 4, 5, IIIrd antennal segment equal to combined lengths of segments XII-XVI
- P. arboris.**
- Small non deciduous spine on distal segment of superior clasper not proximal to all other spines (Plate XXIII, figs 38, 39, 41, 42)
- 20
- 20 Proximal segment of superior clasper about twice length of distal segment, spines comparatively close together* (Plate XXIII, figs 38, 39), ratio α over β more than 2 buccal armature with about 10 separate teeth and pigmented area well developed (Plate XXIII, fig 40) (vide also No 29)
- P. malabaricus.**
- Proximal segment of superior clasper less than twice length of distal segment, spines widely separated (Plate XXIII, figs 41, 42)
- 21

* As the spines are not so widely separated as in the other species, *P. malabaricus* has been given in two places in the table (vide No 29)

- 21 Proximal spines on distal segment of superior clasper arise about middle of segment (Plate XXIII fig 41), ratio α over β less than 0.5, buccal armature unknown

P sylvestris.

Proximal spines on distal segment of superior clasper arise about $\frac{1}{3}$ rd length from apex (Plate XXIII fig 42) ratio α over β more than 0.5, about 14 separate buccal teeth and small linear pigmented area (Plate XXIII, fig 43)

P purii

- 22 With all 4 spines near apex of distal segment of superior clasper almost equally developed (Plate XXIII, figs 45, 51, 54 Plate XXIV, figs 57, 61, 64, 67, 68, 70, 73)

23

With 3 large and 1 small spine near apex of distal segment of superior clasper* (Plate XXIII, fig 47), single row of about 40 buccal teeth with very small pigmented area (Plate XXIII, fig 48), ratio α over β about 2.5

P himalayensis

- 23 Intromittent organ stout with broadish end (Plate XXIII, figs 44, 51, 54)

24

Intromittent organ slender with narrow end (Plate XXIV, figs 59, 62, 65, 67, 68, 70, 73)

26

- 24 Distal segment of superior clasper slender with long thin spines (Plate XXIII, fig 45), buccal teeth about 15, arranged in row markedly concave backwards, pigmented area absent (?) (Plate XXIII, fig 46), wing very narrow, pointed and lanceolate

P. dentatus.†

Distal segment of superior clasper stouter with coarser spines, intromittent organ broader (Plate XXIII, figs 51, 54)

25

- 25 Intermediate appendage with hooked end, non deciduous spine on superior clasper not markedly distal (Plate XXIII, fig 51) pharynx markedly expanded posteriorly (Plate XXIII fig 50) buccal teeth smaller and more numerous (Plate XXIII, fig 49) third antennal segment short (about 90 μ) and only about $\frac{1}{2}$ length of proboscis (Plate XXIII fig 56)

P antennatus ‡

* The small spine on *P himalayensis* may be hidden behind the other spines and so be difficult to observe. For this reason, Annandale (1910a) and Sinton (1924b) described this species as having only 3 spines, although the latter author suspected a fourth.

† Perfiljew (1933) has described a new variety of sandfly from Turkmenistan as *P minutus* var *arpallensis*. This variety appears to be identical with *P dentatus* Sinton 1933. Recently Lieut Colonel H. E. Shortt M.S., collected a female *Phlebotomus* on the Chitral Road between Malakand and Dir N. W. F. Province. This specimen appears to be *P dentatus*, but shows a faint and large pigmented area like the segment of an orange, while in the original specimens from Quetta no such area was found.

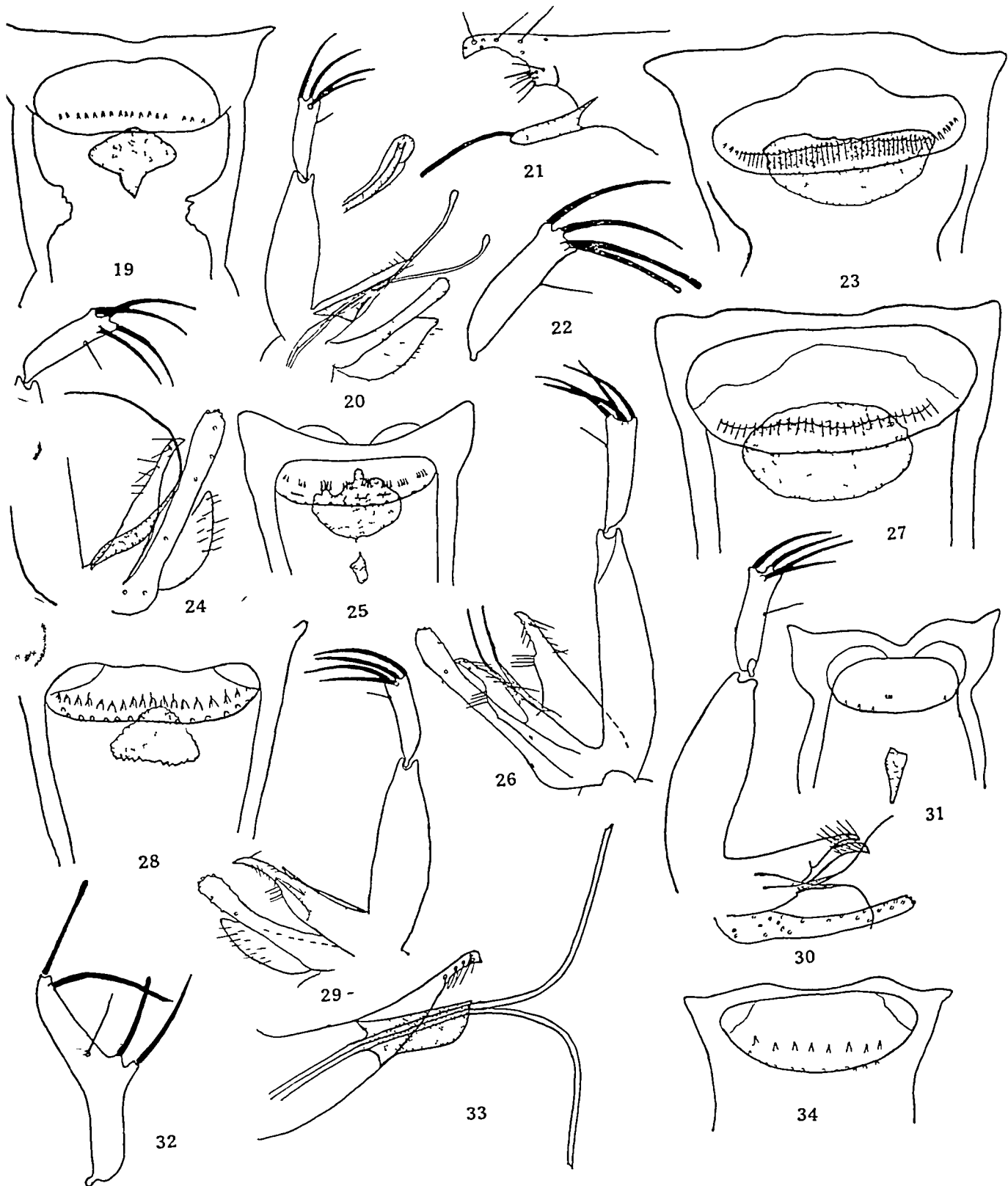
‡ This species was originally described by Newstead (1912) as a distinct species, but Newstead and Sinton (1921) considered the evidence then available was insufficient to separate it from *P minutus* and placed it as a variety of that species. The differences both in the male and female forms of Indian specimens of these two insects are so distinctive that it is now considered that *P antennatus* should be raised again to specific rank. Whether the Indian form of this species is identical with the original African type will require to be investigated. If not, the name *P punjabensis* is proposed for this species, as it seems most prevalent in the Punjab.

- Intermediate appendage with blunt end, non deciduous spine on superior clasper markedly distal (Plate XXIII, fig 54), pharynx not markedly expanded posteriorly (Plate XXIII, fig 52), buccal teeth larger (Plate XXIII fig 53), IIIrd antennal segment longer (about 120 μ) and more than $\frac{1}{2}$ length of proboscis (Plate XXIII, fig 55)
- P. minutus.**
- 26 Genital filament with narrow end (Plate XXIV, figs 59, 68, 73), buccal cavity without marked lateral protrusions (Plate XXIV, figs 57, 60, 63, 66, 69, 71, 72) 27
- Genital filament with bulbous end (Plate XXII, fig 20), buccal cavity with marked lateral inward projections (Plate XXII, fig 19) (*vide* No 12)
- P. squamipleuris.**
- 27 Intromittent organ with pointed end (Plate XXII, fig 29, Plate XXIV, fig 59) 28
- Intromittent organ with narrow rounded or nipple like end (Plate XXIV, figs 62 65, 67, 68, 70, 73) 29
- 28 With row of about 20 separate buccal teeth and distinct pigmented area (Plate XXII, fig 28), antennal formula 1 over IV to XV (*vide* also No 16)
- P. eadithae.**
- With row of about 25-30 approximated buccal teeth, and pigmented area usually absent (Plate XXIV, fig 57), antennal formula 1 over III to VI
- P. africanus.**
- 29 Ratio α over β more than 2 (*vide* also No 20) **P. malabaricus** 30
- Ratio α over β about 1 or less
- 30 IIIrd antennal segment long (not less than 0.2 mm and usually more), ratio α over β not less than 0.75 (Plate XXIV, fig 74), δ usually large 31
- IIIrd antennal segment short (not more than 0.18 mm and usually less), ratio α over β less than 0.75, δ small
- P. babu, P. baghdadis, P. shortii ***
- 31 Pigmented area very poorly developed, usually absent (Plate XXIV, figs 60, 63) 32
- Pigmented area distinctly developed and several rows of lateral buccal teeth (Plate XXIV, fig 66)
- P. montanus.**
- 32 With single regular row of 16-20 separate buccal teeth (Plate XXIV, fig 60), spines of distal segment of superior clasper not markedly sub apical, small non deciduous spine distinctly proximal to other spines (Plate XXIV, fig 61), ratio α over β about 1 **P. barraudi.**
- With several rows of small and irregularly dispersed buccal teeth (Plate XXIV, fig 63), 2 spines on distal segment of superior clasper markedly sub apical, and non deciduous spine almost at same level (Plate XXIV, fig 64), ratio α over β less than 1 **P. bailyi**

* These three species are very closely allied and the males are often impossible to distinguish. The geographical distribution of *P. shortii* on the Eastern Frontier of India and in Burma, of *P. baghdadis* in the northern and western areas and of *P. babu* intermediate, helps. In *P. shortii* the distal segment of the superior clasper is relatively shorter and stouter and δ is usually very small, often negative. The number of buccal teeth helps in some instances (*cf* Plate XXIV, figs 69, 71, 72)

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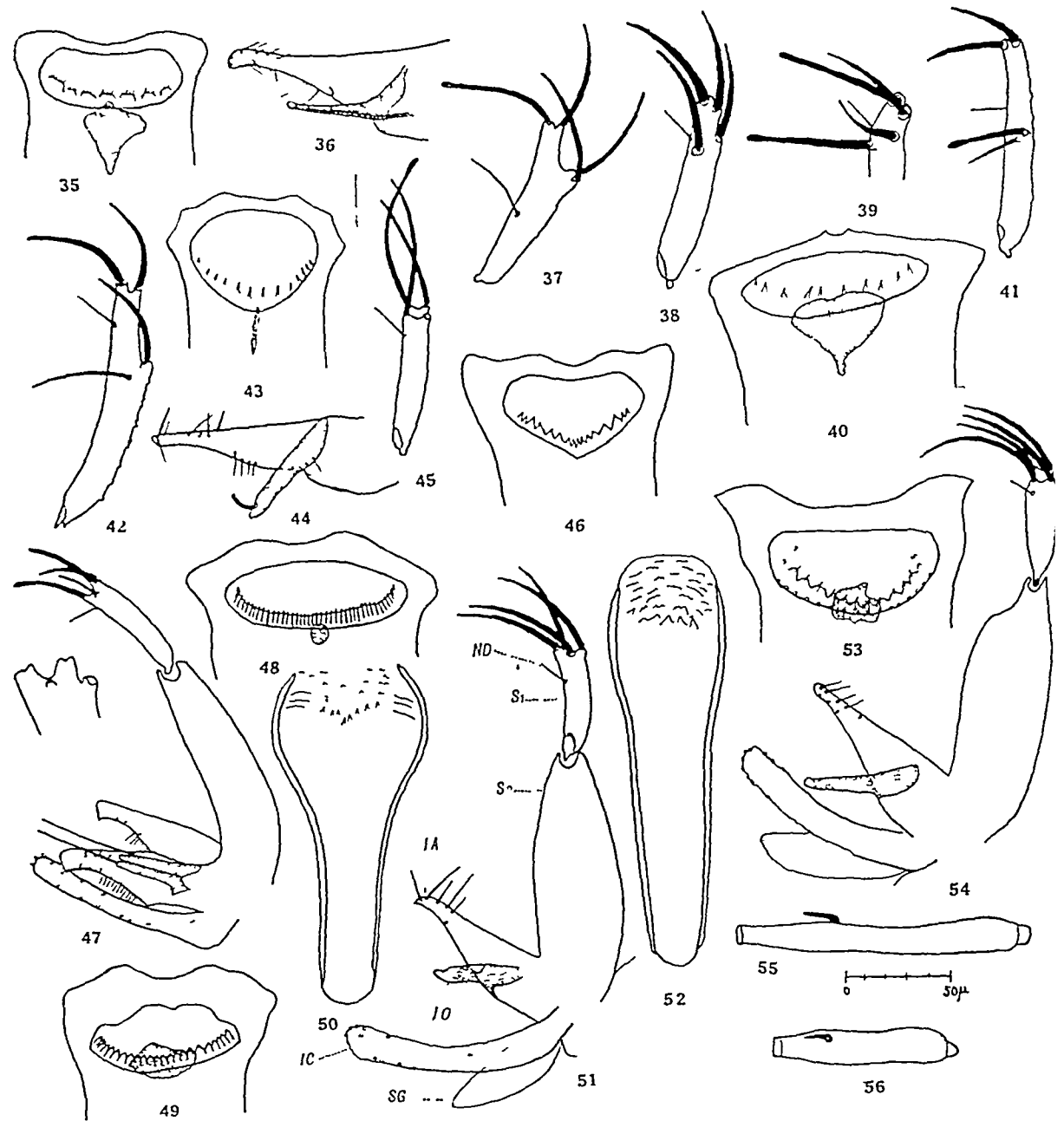
Fig 1 Hypopygium of *P. colabansis* Fig 2 Hypopygium of *P. maynei* Fig 3 Hypopygium of *P. sergenti*
 Fig 4 Distal segment of superior clasper of *P. sergenti* var. *alexandri* Fig 5 Brush on proximal segment
 of superior clasper of *P. sergenti* var. *alexandri* Fig 6 Dorsal surface of IInd abdominal segment of *P. christophersi*
 showing scars of hairs E—scars of erect hairs R—scars of recumbent hairs Fig 7 Wing of *P. sergenti* var.
alexandri (note size as compared with fig 8) Fig 8 Wing of *P. sergenti* (sens. restr.) Fig 9 Hypopygium
 of *P. papatasi* Fig 10 Hypopygium of *P. argentipes* Fig 11 Hypopygium of *P. newsteadii* Fig 12
 Halter of *P. papatasi* (compare with fig 13) Fig 13 Characteristic halter of *P. newsteadii* Fig 14 Hypo-
 pygium of *P. eleanorae* Fig 15 Wing of *P. papatasi* Fig 16 Wing of *P. argentipes* Letters show different
 parts of venation (vide Appendix I) Fig 17 Hypopygium of *P. major*, showing end of intromittent organ
 (lettering explained in Appendix I) Fig 18 Hypopygium of *P. chinensis*, showing end of intromittent organ



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Fig 19 Buccal cavity of *P. squamipleuris* Fig 20 Hypopygium of *P. squamipleuris*, showing dilated end of genital filament Fig 21 Intermediate appendage and intromittent organ of *P. hodgsoni* Fig 22 Distal segment of superior clasper of *P. hodgsoni* Fig 23 Buccal cavity of *P. hodgsoni* Fig 24 Hypopygium of *P. clydei* Fig 25 Buccal cavity of *P. clydei* Fig 26 Hypopygium of *P. hospiti* Fig 27 Buccal cavity of *P. hospiti* Fig 28 Buccal cavity of *P. eadithae* Fig 29 Hypopygium of *P. eadithae* Fig 30 Hypopygium of *P. christophersi* Fig 31 Buccal cavity of *P. christophersi* Fig 32 Distal segment of superior clasper of *P. zeylanicus* Fig 33 Intermediate appendage and intromittent organ of *P. zeylanicus* Fig 34 Buccal cavity of *P. zeylanicus*

PLATE XXIII



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Fig 35 Buccal cavity of *P. arboris* Fig 36 Intermediate appendage and intramittent organ of *P. arboris*
 Fig 37 Distal segment of superior clasper of *P. arboris* Fig 38 and Fig 39 Distal segment of superior clasper
 of *P. malabaricus* Fig 40 Buccal cavity of *P. malabaricus* Fig 41 Distal segment of superior clasper of
P. sylvestris Fig 42 Distal segment of superior clasper of *P. puri* Fig 43 Buccal cavity of *P. puri* Fig 44 In
 termediate appendage and intramittent organ of *P. dentatus* Fig 45 Distal segment of superior clasper of *P. dentatus*
 (one spine is missing) Fig 46 Buccal cavity of *P. dentatus* Fig 47 Hypopygium of *P. himalayensis* Fig 48
 Buccal cavity of *P. himalayensis* Fig 49 Buccal cavity of *P. antennatus* Fig 50 Pharynx of *P. antennatus*
 Fig 51 Hypopygium of *P. antennatus* (lettering explained in Appendix I) Fig 52 Pharynx of *P. minutus* Fig 53
 Buccal cavity of *P. minutus* Fig 54 Hypopygium of *P. minutus* Fig 55 IIIrd antennal segment of *P. minutus*
 Fig 56 IIIrd antennal segment of *P. antennatus*



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Fig 57 Buccal cavity of *P africanus* Fig 58 Distal segment of superior clasper of *P africanus* Fig 59 Intermediate appendage and intromittent organ of *P africanus* Fig 60 Buccal cavity of *P barraudi* Fig 61 Distal segment of superior clasper of *P barraudi* Fig 62 Intermediate appendage and intromittent organ of *P barraudi* Fig 63 Buccal cavity of *P bairdi* Fig 64 Distal segment of superior clasper of *P bairdi* Fig 65 Intermediate appendage and intromittent organ of *P bairdi* Fig 66 Buccal cavity of *P montanus* Fig 67 Hypopygium of *P montanus* Fig 68 Hypopygium of *P baghdadis* Fig 69 Buccal cavity of *P baghdadis* Fig 70 Hypopygium of *P babu* Fig 71 Buccal cavity of *P babu* Fig 72 Buccal cavity of *P shortii* Fig 73 Hypopygium of *P shortii* Fig 74 Wing of *P montanus* Fig 75 Wing of *P shortii*

APPENDIX I

EXPLANATION OF TERMS USED IN THE TABLE

A *Erect and recumbent abdominal hairs in classification*

These conditions have already been explained in the appendix to the diagnostic table for females (Sinton, 1932a)

The occurrence of such hairs is closely related to the morphology of the spermathecae of the females and has been used in the classification of *Phlebotomus* (Sinton, 1927d, 1927e, 1927f, 1929b) Nitzulescu (1931) remarks, however, that 'cette division, malgré son apparence séduisante, n'est pas satisfaisante, car le caractère de la disposition des poils ne possède pas toute netteté désirable' He instances the case of *P. malabaricus*, which was described by Sinton (1924c) as having 'slightly semi-recumbent hairs' on the abdomen, although the spermathecae of the female of this species (?)* are not segmented As mentioned previously in this paper, it is now known that the abdominal hairs are very liable to displacement in dry specimens and that the only true criterion of the original condition is that of the scars left by the detachment of the hairs The scars in the female of *P. malabaricus* (?)* show it to belong to the recumbent-haired group and the discrepancy mentioned can thus be explained

Nitzulescu (1931) in his sub-genera *Brumptius* and *Phlebotomus* has grouped together a large number of species with very varied characters It seems to me that these sub-genera are artificial and will require further division in future

B *Antennæ, palps, buccal cavity and pharynx*

The terms used in connection with these structures have already been explained (Sinton, 1932a)

C *Wing venation*

França and Parrot (1920) suggested that for ease of description certain letters of the Greek alphabet should be used to denote the lengths of certain portions of the wing venation This method was extended by Sinton (1923a) and has been widely adopted in technical descriptions of these insects

The letters used in the above table have been illustrated in Plate XXI, fig. 16

α —The length of the anterior branch of the distal fork of the 2nd longitudinal vein

β —The distance between the two forks of the 2nd vein

γ —The distance between the proximal fork of the 2nd vein and the cross vein which unites the stem of this vein with the 3rd

δ —The distance by which the termination of the 1st vein surpasses or falls short of the distal fork of the 2nd In the latter instance this length is preceded by the negative sign

* It is also still doubtful whether the type male described by Sinton (1924c) is the same species as the recumbent haired female described later as *P. malabaricus* (?) (Sinton, 1927a)

- 11 *P chinensis* —Adler and Theodor (1929a), Newstead (1916), Nitzulescu (1930a), Sinton (1929b, 1932a, 1933d), Sinton and Barraud (1928)
- 12 *P christophersi* —Sinton (1927d, 1927e, 1927f, 1927g, 1929b, 1932a, 1933d)
- 13 *P clydei* —Sinton (1928b, 1932a, 1933d)
- 14 *P colabaensis* —Sinton (1932a, 1933d), Young and Chalam (1927)
- 15 *P demeigerei* (see *P sylvestris*) —Nitzulescu (1930b), Sinton (1931e)
- 16 *P dentatus* —Peifiljew (1933), Sinton (1933a, 1933d)
- 17 *P eadithæ* —Sinton (1932b, 1933d)
- 18 *P cleanoiæ* —Sinton (1931a)
19. *P himalayensis* —Annandale (1910a), Brunetti (1912), Sinton (1924a, 1924b, 1932a)
- 20 *P hodgsoni* —Sinton (1933b, 1933d)
- 21 *P hospiti* —Sinton (1924g, 1927e, 1927f, 1929b, 1932a)
- 22 *P vyengari* —Sinton (1933c, 1933d)
- 23 *P major* —Adler and Theodor (1929a), Annandale (1910a), Brunetti (1912), Sinton (1924a, 1927e, 1929b, 1932a), Sinton and Barraud (1928)
- 24 *P malabaricus* —Annandale (1910a), Brunetti (1912), Sinton (1924a, 1924c, 1927a, 1927e, 1927f, 1932a)
- 25 *P maynei* —Sinton (1930b)
- 26 *P minutus* * —Adler and Theodor (1926, 1927, 1929), Sinton (1932a)
- 27 *P montanus* —Sinton (1924a, 1927d, 1927e, 1927f, 1929b, 1932a)
- 28 *P newsteadæ* —Sinton (1926a, 1928a, 1929b, 1932a)
- 29 *P papatasii* —Adler and Theodor (1926), Annandale (1910a), Brunetti (1912), Grassi (1907), Newstead (1911), Sinton (1924a, 1927e, 1929b, 1932a), Sinton and Barraud (1928)
- 30 *P perturbans* (see *P sylvestris*) —Annandale (1910a), Brunetti (1912), Sinton (1924a, 1924d)
- 31 *P puri* —Sinton (1931c, 1932a)
- 32 *P sergenti* —Adler and Theodor (1929a), França (1918), Newstead (1920), Parrot (1917), Sinton (1924a, 1929b, 1932a), Sinton and Barraud (1928)
- 32a *P sergenti* var *alexandri* —Newstead (1920), Sinton (1928c, 1932a)
- 33 *P shortti* —Adler and Theodor (1927), Sinton (1932a)
- 34 *P squamipleuris* —Newstead (1912), Sinton (1923b, 1924a, 1927c, 1927d, 1927e, 1927f, 1929b, 1932a, 1933d), Theodor (1931)

* The species now commonly known as *P minutus* is that described by Adler and Theodor (1926, 1927) from Palestine. It is doubtful whether this is the same as *P minutus* Rondani, 1843, from Italy. The latter may be synonymous with *P parroti* Adler and Theodor, 1927.

- 35 *P. stantoni* —Newstead (1914), Sinton (1923*a*, 1931*d*, 1933*d*)
 36 *P. sylvestris* —Annandale (1910*a*), Brunetti (1912), Nitzulescu (1930*b*),
 Sinton (1924*a*, 1924*d*, 1927*d*, 1932*a*)
 37 *P. zeylanicus* —Annandale (1910*b*), Brunetti (1912), Newstead (1914),
 Sinton (1921*f*, 1932*a*), Young and Chalam (1927)

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OBSERVATIONS ON THE CHEMISTRY OF THE OXYTOCIC HORMONE OF THE PITUITARY GLAND

Part I

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SINCE the recognition of the presence of an oxytocic principle in the posterior lobe of the pituitary gland, some work has been done to isolate it and to throw light on its chemical nature. The controversy about the identity or otherwise of the oxytocic and pressor principles of the pituitary gland has now been settled by a fairly effective separation of the two hormones (Kamm *et al* , 1928). The work of Kamm *et al* has facilitated a renewed attack on this problem, as it is now possible to obtain a preparation rich in the oxytocic principle which is relatively free from pressor activity.

The object of the present investigation was to obtain information about the general chemical behaviour of the oxytocic principle, so that a method for its concentration and eventual separation might be applied with advantage. The earlier work on the subject (Aldrich, 1908, Engeland and Kutscher, 1911-1912, Fuhner, 1913, Abel and Pincoffs, 1917, Abel and Kubota, 1919, Dudley, 1919) indicates that the oxytocic principle is of the nature of either a base or peptide. Kamm *et al* (1928) suggest that the hormone is probably a base, while the recent work of Freudenberg, Weiss and Eyer (1932) and of Gulland and Macrae (1933) on the inactivation of relatively purified preparations of the oxytocic hormone by enzyme preparations points to the possibility of the hormone possessing a peptide structure. Gulland and Macrae further point out that the inactivating enzyme concerned accompanies preparations of dipeptidase, amino-polypeptidase and papain, but is not identical with any of these.

In this paper we have reported the action of various reagents, like nitrous acid, benzoyl chloride, phospho-tungstic acid, picrolonic acid, etc., on the oxytocic principle. Some work has also been carried out to investigate the heat-stability of our preparations at various hydrogen ion concentrations. We have found the active principle to be most stable in the region between pH 3.0 and pH 5.0, which is in general agreement with the observations of Gaddum (1930). We have, however, found our preparation to be more stable in the alkaline region than the earlier work of Guggenheim (1914) would indicate. It is likely that some discrepancies in this respect might be related to the presence of accompanying substances as is known to occur in the cases of vitamins B₁ and B₂ (Guha and Drummond, 1929, Guha, 1931). It is also possible that the presence of small amounts of histamine as an impurity in our preparations might be the cause of their relative stability. This point is receiving further attention, although the contamination by histamine of these preparations does not appear to be considerable. After this work was completed, an observation of the action of nitrous acid on the oxytocic hormone has been published by Gulland (1933) which indicates that the activity is diminished but not entirely abolished by nitrous acid. Under our conditions of experiment the preparations have been found to be more stable to nitrous acid.

Technique of oxytocic assay

The potency of the final products was tested by the usual method with slight modifications (Dale and Laidlaw, 1912, Burn and Dale, 1922). The comparison was made by finding the suitable dose which could produce a contraction in a virgin guinea-pig's uterus equal to that produced by the standard chosen. The standard dose, however, was always selected so as to produce only sub-maximal contraction of the uterus. Standard doses were always given at the beginning and at the end of the experiment.

PREPARATION OF THE MATERIAL

Three hundred and thirty fresh posterior lobes of bovine pituitary glands, which were kept under acetone were dissected and dried by means of an electric fan. The dry glands weighed 15 g. These were then worked up by the general procedure described by Kamm *et al* (1928).

The glands were ground up with dry washed sea-sand and were heated to boiling with 619 c.c. of 0.25 per cent acetic acid. Heating was so regulated that the temperature went up to 95°C within 20 minutes. It was then cooled immediately in ice-cold water. When the temperature came down to about 20°C, the extract was filtered under pressure. The residue was re-extracted with 1/10 the original amount of acidulated water.

The combined filtrates were then evaporated *in vacuo* to a small volume (60 c.c.). Sixty g. of dry (NH₄)₂SO₄ were added. The vessel was left in the refrigerator overnight and next day the precipitate was collected by filtration under suction. The clear filtrate had no oxytocic activity.

The residue weighing 23 g, after having been dried in the steam oven at 40°C , was triturated with glacial acetic acid and warmed. The acid extract was collected by filtration under suction and this process was repeated thrice. To the total acid extract was then added a mixture of petrol ether and ethyl ether. A precipitate soon formed and the whole was kept overnight in the refrigerator.

The supernatant ethereal layer was decanted off and the precipitate, which stuck to the bottom of the vessel, was washed acid-free with ether mixture. The precipitate was finally dried by means of a fan and was taken up in warm water and filtered.

In all the subsequent investigation this, or a preparation similarly obtained, served as the starting material.

EXPERIMENTAL

Heat-stability of the oxytocic hormone of the pituitary gland

From a few preliminary experiments the hormone appeared fairly stable to mild heat treatment even in alkaline regions, so it was considered desirable to observe the limit of its stability. The following experiment was accordingly performed —

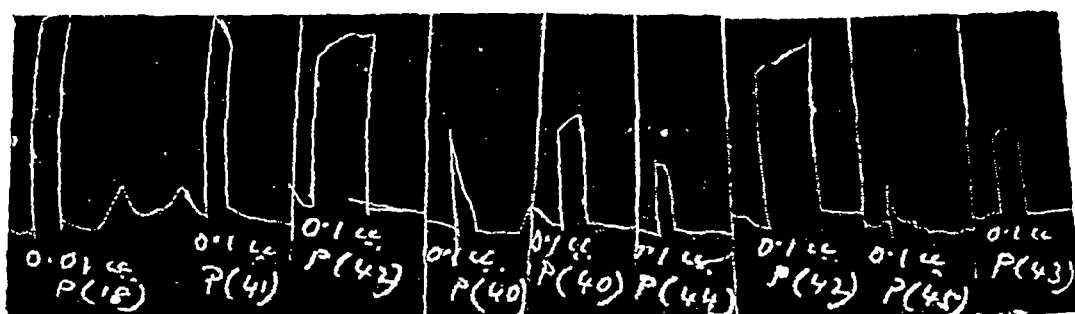
In each of six small Erlenmeyer flasks was taken 1 c.c. of the extract, which was made up to 4 c.c. with distilled water. The contents of the flasks were then brought to pH 1.0, 3.0, 5.0, 7.0, 9.0 and 11.0 respectively. They were then autoclaved simultaneously for 1 hour under a pressure of 1 atmosphere. All the solutions were then cooled and the shift of pH noted. The pH of the alkaline solutions was, however, immediately adjusted to 5.0. All the solutions were then made up to a definite volume (10 c.c.) and assayed. The shift of pH was as follows —

Initial pH	Final pH after autoclaving
1.0	2.0
3.0	5.0
5.0	5.2
7.0	7.0

Table I gives the results of the experiment (see also text-figure) P (18) represents the original pituitary extract

TABLE I

Number of preparation	pH at which the solution was autoclaved	Percentage of inactivation
P (40)	1 0	70
P (41)	3 0	50
P (42)	5 0	60
P (43)	7 0	75
P (44)	9 0	above 80
P (45)	11 0	almost 100



Text figure

The chemistry of the hormone

1 *Treatment with H_2O_2* —One c c of the extract [P (9)] was treated with 1 c c of a solution of H_2O_2 in water (Merck's, 12 per cent by volume) The excess of H_2O_2 was ensured by testing with starch-iodide The mixture was warmed on water-bath for 20 minutes and finally boiled for 2 to 3 minutes in an open beaker with 5 c c of water added It was then made up to 8 c c [P (10)]

One c c of P (9) was made up to about 8 c c with distilled water and boiled for 4 minutes in an open beaker as a control, and made up to 8 c c [P (11)]

2 *Treatment with bromine*—Two c c of the extract were treated with 4 to 5 drops of bromine On shaking precipitation took place and the supernatant liquid became reddish On filtering under suction, a very small amount of residue remained on the filter-paper It was then washed with $\frac{1}{2}$ to 1 c c water

The filtrate was left in a vacuum desiccator over $CaCl_2$ and soda-lime When dry, it was made up to a definite volume and was tested [P (16)]

3 *Treatment with HNO_3* —Two c c of the extract were treated with 5 to 6 drops of concentrated HNO_3 (Kahlbaum, A R) The solution became milky but

on the addition of one or two more drops of the acid the precipitate dissolved. The milkiness reappeared on cooling in ice. The milky solution was filtered under suction. The liquid was allowed to evaporate in the vacuum desiccator over CaCl_2 and soda-lime. The substance left was dissolved in water to a definite volume and tested [P (17)].

1 *Benzoylation of the pituitary extract*—To 2 c.c. of the pituitary extract were added 1.5 c.c. of benzoyl chloride and about 7 c.c. of 20 per cent NaOH . The reaction vessel was thoroughly shaken. The vessel became hot. No appreciable quantity of precipitate formed. The vessel was corked and left in the refrigerator to settle.

The mixture was repeatedly extracted with ether and the aqueous portion was then decomposed with concentrated HCl till the reaction of the mixture was slightly acid. The benzoic acid which separated out, was removed by filtering under suction and any benzoic acid in the solution was removed by repeated extraction with ether. The final aqueous solution was made up to a definite volume and tested [P (15)].

5 *Treatment with SO_2* —Two c.c. of the extract were diluted to 5 c.c. and cooled in ice. Washed SO_2 from a generator ($\text{Cu} + \text{H}_2\text{SO}_4$) was passed into the cold solution for about 15 minutes. The solution, saturated with SO_2 , was then kept at the room temperature for half an hour to allow the dissolved SO_2 to react. White flakes appeared as the treatment with SO_2 began. The solution was evaporated to dryness in a vacuum desiccator over CaCl_2 and soda-lime. Finally, the solution was made up to a volume of 2 c.c. and was tested for activity [P (22)].

6 *Treatment with HNO_3* —To 5 c.c. of the extract were added about 4 drops concentrated HCl and 12 to 15 drops of 50 per cent NaNO_2 with stirring. The excess of HNO_3 in the solution was ensured by testing with starch-iodide. While adding NaNO_2 solution, a precipitate formed. After the reaction was over, the mixture was heated over a water-bath for about 5 minutes, cooled and finally evaporated to dryness in a vacuum desiccator over CaCl_2 and soda-lime. When dry the residue was taken up in 5 c.c. of water and tested [P (12)].

Two controls were made: one with 5 c.c. of distilled water, 4 drops of concentrated HCl and 15 drops of 50 per cent NaNO_2 , heating over a water-bath for 5 minutes, and drying in the desiccator [P (12a)], and another with 2 c.c. of the extract and 2 drops of concentrated HCl , heating for 5 minutes over a water-bath and drying in the same desiccator [P (12b)]. Volumes of these controls were made equal to their original volumes and tested.

7 *Treatment with basic precipitants*—Some workers (*vide supra*) have given evidence as to the basic nature of the hormone, although none of it may be regarded as conclusive. Following upon such observations, some basic precipitants were tried to see whether the active material could be completely precipitated by these. The results were not very encouraging.

(a) *Precipitation with phospho-tungstic acid*

Five c.c. of the extract were treated with 3 c.c. of saturated phospho-tungstic acid solution in 5 per cent H_2SO_4 and were left overnight in the refrigerator.

A white amorphous precipitate settled to the bottom. The precipitate was filtered under suction and washed with a little water.

Both the filtrate and precipitate were decomposed with saturated baryta solution, barium sulphate and phospho-tungstate removed by filtration under suction, and excess of barium precipitated by adding requisite amount of dilute H_2SO_4 . The barium sulphate precipitate was removed by centrifugation and the solutions kept slightly acid.

The phospho-tungstic acid filtrate fraction measured finally 53 c c and was numbered P (32a).

The precipitate fraction measured 45 c c and was numbered P (33a).

(b) Precipitation with picrolonic acid

One c c of the extract was treated with 0.5 c c (excess) of an alcoholic solution of picrolonic acid and left in the cold store. A yellow crystalline precipitate came down and was filtered and washed with a little water. Both the precipitate and the filtrate were decomposed by a few drops of concentrated HCl and extracted repeatedly with ether. The aqueous layers were nearly neutralized with $\text{N}/5 \text{ NaOH}$.

The precipitate fraction measured 6 c c and was numbered P (38).

The filtrate fraction which measured 7 c c was numbered P (39).

8 *Adsorption experiments*—(a) Adsorption of the oxytocic principle with fuller's earth. Five c c of the extract were brought to pH about 4.5 by adding a few drops of dilute HCl and agitated with 0.25 g of fuller's earth (Merck's) for 15 minutes. This was filtered under suction and the clear filtrate [P (19)] was tested for oxytocic activity.

(b) The clear filtrate from the above experiment was agitated with 0.25 g of kieselguhr for 10 minutes, centrifuged and the clear centrifugate was tested for oxytocic activity [P (20)].

(c) Two c c of the extract were diluted to about 4 c c and were then brought to pH 1.5 by adding HCl . 0.28 g of fuller's earth (Merck's) was stirred into the extract and the mixture agitated for about 15 minutes and filtered under suction. The clear filtrate was tested for activity [P (23)].

The results of the tests are summarized in Tables II and III —

TABLE II

Number of preparation	Reaction	Percentage inactivation
P (10)	H_2O_2	33
P (11)	Heating for 4 minutes over an open flame	0
P (12)	HNO_2	0
P (12a)	Control with HNO_2	0
P (12b)	"	0
P (16)	Bromine	60
P (17)	HNO_3	0
P (22)	SO_2	50

TABLE III

Number of preparation	Fraction tested	Percentage of activity retained in fraction tested
P (15)	Aqueous fraction obtained after benzoylation	50
P (19)	Filtrate from adsorption with fuller's earth at pH 4.5	100
P (20)	Filtrate from adsorption with kieselguhr at pH 4.5	100
P (23)	Filtrate from adsorption with fuller's earth at pH 1.5	100
P (32a)	Phosphotungstic acid filtrate	Activity distributed between the filtrate and precipitate P (32a) P (33a) = 1 : 1.75
P (33a)	Phosphotungstic acid precipitate	
P (38)	Picrolonic precipitate	50
P (39)	Picrolonic filtrate	50

SUMMARY

1 The stability of preparations of the oxytocic hormone of the pituitary gland at pH 1.0, 3.0, 5.0, 7.0, 9.0 and 11.0 to heating at 1 atmosphere pressure for 1 hour has been studied. The principle appears to be most stable between pH 3.0 and 5.0.

2 The behaviour of the hormone towards nitric acid, nitrous acid, bromine, hydrogen peroxide, sulphur dioxide, benzoyl chloride, acetyl chloride, picrolonic acid and phosphotungstic acid has been studied.

3 The hormone could not be adsorbed by fuller's earth at pH 4.5 and 1.5 or by kieselguhr at pH 4.5.

We are indebted to Dr B. B. Chatterji for his generous help at the preliminary stages of this work.

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OBSERVATIONS ON FILARIASIS IN SOME AREAS IN BRITISH INDIA

Part IX

SIND AREA

BY

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IN continuation of the inquiry into the distribution of filarial infection which has been carried out in different parts of India presenting different geographical and climatic conditions, observations on filariasis were made in the jails of Larkana, Sukkur and Shikarpur in Sind in the months of September and October 1932 and in February 1933, respectively

The blood material for this investigation was derived from 500 persons in the above jails, out of which 446 persons had their residential addresses in the alluvial tract of Sind

The thick blood smears were taken for the purpose by the Officer-in-Charge Sind Malaria Inquiry and examined by me. The material was taken during the night hours of 8 p.m. and 9 p.m.

A PHYSICAL ASPECTS OF THE SIND AREA (*Imp Gaz India*, 1908)

The province of Sind has an area of 53,116 square miles and a population (1901) of 3,410,223 (including the state of Khairpur)

The province is essentially a desert one with a central area along the banks of the Indus about 160 miles from north to south, fertile and well cultivated, which is likely to be greatly extended as the result of irrigation from Sukkur barrage. This area includes Larkana, Shikarpur and Sukkur (the places from which the blood specimens for the present investigation were obtained), and Jacobabad in the north to Hyderabad, Jamesabad and Umarkot (Thar Parkar) in the south (McCombie Young and Majid, 1930 Map 1)

The conditions in the hilly tracts to the west and the desert areas to the east were not dealt with

Climatic conditions—Sind is a country of extremes of temperature, especially in the north away from the sea coast. Hyderabad and Jacobabad are notorious for recording annually some of the highest temperatures reached in India, and this along with extreme dryness. On the other hand, during the cold weather the night temperature is frequently below freezing point and in the day time it may again rise as high as 80°F. The average of the hot weather months is 95°F with occasional maximums up to 120°F, while the average of the winter months is 60°F. The hot weather is more prolonged than in any other part of India. The mean relative humidity is low, and reaches its highest point in August and September at Sukkur. It ranges between 41 per cent in April to 78—77 per cent in August and September respectively (McCombie Young and Majid, *loc cit*).

In northern Sind, the areas dealt with, the influence of the monsoon is slight. The rainfall is scanty and irregular and averages only from 3 to 5 inches, (McCombie Young and Majid, *loc cit*). But for the fact that the Indus provides water for the irrigation of a large area all this part of Sind would be a desert. The type of cultivation is similar to that in other desert areas which have the advantage of river irrigation. Out of total of 47,066 square miles (British Sind) only 6,444 are cultivated (1903-1904).

Bajra and *jowar* are the two chief food-grains in Sind. Larkana district is a rice-producing area and Sukkur a 'dry crop' area. The area in square miles under each staple crop in 1903-1904 was as follows—

Jowar (1,051), *bajra* (1,478), rice (1,381), wheat (858) and other food-stuffs (765).

In the more fertile alluvial areas, Larkana shows a population of 129 per square mile, Hyderabad 119, Sukkur 97, while in the desert area of Khairpur State and Thai Parkar districts the figures are respectively 33 and 27.

The area dealt with accordingly shows a marked contrast in climatic and physical conditions with most of the other areas of India which have been surveyed in connection with the present series of investigations.

Extremes of temperature with marked aridity are the characteristic features of the climate, and desert conditions with a limited area watered by the Indus, are the main physiographical characteristics.

Culex fatigans

The Entomologist, Malaria Survey of India, reports that *C. fatigans* is common in Sind and in most districts in North-West India.

B EXAMINATION FOR FILARIASIS

The blood of 500 persons examined at night between the hours of 7 p.m. and 9 p.m. in the jails of Larkana, Sukkur and Shikarpur, showed uniformly negative results. Of the examined, 54 came from other areas in India and 446 were permanent residents of Sind.

The permanent residents were distributed as follows —

District areas (plain tract)	Total examined
Bahawalpur	10
Dadu	45
Hyderabad	5
Jacobabad	104
Karachi	1
Larkana	73
Nawabshah	14
Shikarpur	2
Sukkur	191
Thar Parkar	1
TOTAL	446

The following figures show the number of persons in relation to age (all males) —

Age in years	Total examined
15-20	11
20-30	118
30-40	297
40-50	37
50-60	37
TOTAL	500

Data as regards the class and type of population are not available

CONCLUSIONS

Examination of the blood of 500 persons taken at night between 7 p m and 9 p m in the male convict population of Larkana, Sukkur and Shikarpur jails, as

observed in relation to the district areas, failed to give any evidence of the incidence of filariasis in the province of Sind

I am indebted to the Director, Malaria Survey of India, and the Officer-in-Charge, Sind Malaria Inquiry, for sending me the blood films for this observation and Captain P J Ballaud for the information regarding the prevalence of *Culex fatigans*

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FURTHER OBSERVATIONS ON VITAMIN A IN INDIAN FISH-LIVER OILS

BY

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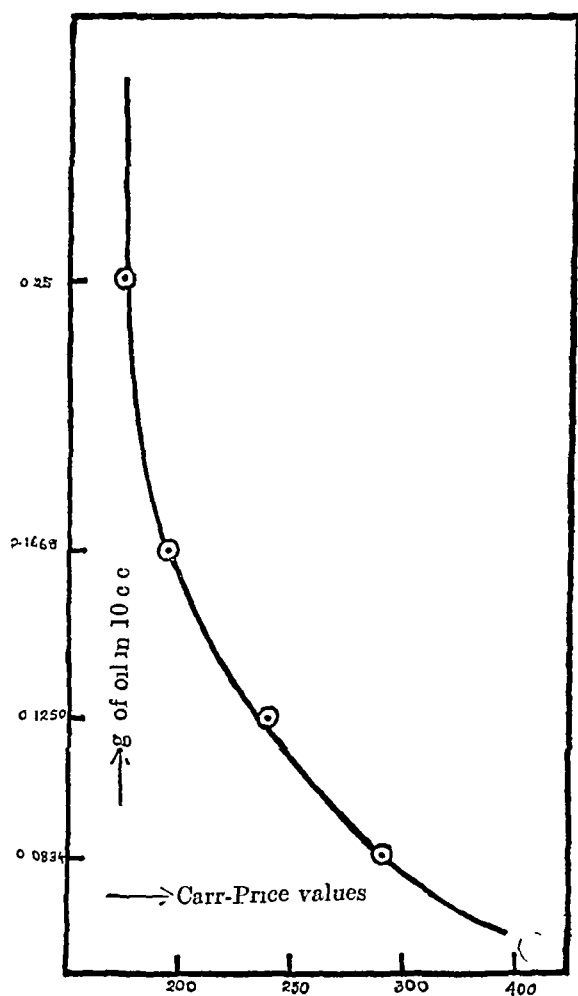
THIS paper records the results of further work on the vitamin A-content of some Bengal fresh-water fishes, which has lately been carried out in this laboratory. In a previous paper (Chakravorty, Mookerjee and Guha, 1933) it was shown that the liver oils of some of the commonly consumed fishes of Bengal are more potent in vitamin A than cod-liver oil, when the measurements are made according to the well-known tintometric technique (Carr and Price, 1926). The same method of assay has been used in the present investigation.

EXPERIMENTAL

Preparation of the material—The liver was triturated with about 10 times its weight of anhydrous sodium sulphate, extracted exhaustively with ether in the cold and the ether evaporated off by means of a fan. The oils obtained were coloured and fairly mobile. Oils were preserved in the refrigerator in sealed brown-glass ampoules in an atmosphere of CO₂. When required for test, the oil was taken out and the ampoule was again filled up with CO₂ and sealed immediately. It is hoped that the deterioration of the vitamin was thus reduced to a minimum.

Technique of the tintometric estimation—Tintometric examinations of the oils were done according to the method described by Carr and Price (*loc cit*) with the Lovibond tintometer of the B D H pattern. Since the oils, in 20 per cent solution, would give very high blue values unsuitable for comparison, the concentration

chosen was much lower. But it has been shown (Chakravorty, Mookerjee and Guha, *loc cit*) that the Carr-Price values calculated from blue readings obtained at lower concentrations are invariably higher than those calculated from readings taken at higher concentrations. It was desirable therefore to find the range in which the Carr-Price value curve would be parallel to the ordinate, as comparison at these dilutions would give more consistent results. Accordingly, a curve was obtained (see Text-figure) for the *dharin*-liver oil and from this the suitable concentrations for finding out the correct Carr-Price values were ascertained.



TEXT FIGURE

The parallelism between the tintometric values and biological values has been questioned repeatedly of late. It appears that liver oils might contain a chromogen besides vitamin A producing the reaction with antimony trichloride (Gillam and Morton, 1931, Ender, 1932, Norris, 1931). We also feel that the Carr-Price values of the oils do not necessarily give the relative biological values. The biological examination of the oils is in progress, and will be reported later.

1 *Chital* (*Notopterus chitala*) liver oil

48 g of *chital* liver gave 2.57 g of a dull yellow mobile oil

0.6 g of oil diluted to 10 c c with anhydrous CHCl_3 was used for tintometric work

This solution gave a very intense coloration, unsuitable for comparison. So, 1 c c of the above solution was further diluted to 10 c c with CHCl_3

Blue units	Yellow units	Neutral tints
6.2	2.5	0.1
6.4	2.5	0.1
6.2	2.3	0.1

Mean blue value

6.2

Carr-Price value

206

2 *Shilong* (*Silumpia silumpia*) liver oil

55 g of the liver yielded 1.95 g of a dull reddish-yellow oil. With chloroform it gave a slightly opalescent solution.

For tintometric work 0.4 g of the oil in 10 c c CHCl_3 was used

Blue units	Yellow units	Neutral tints
15	9.5	
15	8.9	
15	8.9	

Mean blue value

15

Carr-Price value

75

3 *Kalibaus* (*Labeo calbasu*) liver oil

65 g of the liver gave 5.36 g of a pale yellow oil. This oil gave a very turbid solution with CHCl_3 , and consequently the comparison was far from being satisfactory. The relative poorness of the oil in tintometric value also added to the difficulty. The result given is only an approximate one.

Mean blue value

4-5

Carr-Price value

9

444 *Further Observations on Vitamin A in Indian Fish-Liver Oils.*

4 *Dham* (a large variety of *silur* fish) liver oil

8.3 g of a deep reddish yellow oil were obtained from 176 g of the liver

The oil was examined in some detail so that a dilution curve might be obtained

(a) 0.1668 g of the oil in 10 c c

Blue units	Yellow units	Neutral tints
16.1	2	
16.0	2.1	0.1
16.2	2.1	0.1

Mean blue value

16.1

Carr-Price value

193

(b) 0.2501 g of the oil in 10 c c

Blue units	Yellow units	Neutral tints
22.0	3.4	
22.1	3.3	0.1
22.0	3.3	0.1

Mean blue value

22.0

Carr-Price value

175.8

(c) 0.0834 g of the oil in 10 c c

Blue units	Yellow units	Neutral tints
12.0	1.1	0.1
12.2	2.0	0.1
12.2	2.0	0.1

Mean blue value

12.1

Carr-Price value

290

(d) 0.1250 g of the oil in 10 c.c.

Blue units	Yellow units	Neutral tints
14.5	2.4	0.4
15.5	2.4	0.5
14.6	2.4	0.5
14.5	2.4	0.5

Mean blue value

14.8

Carr-Price value

237

5. *Ar* (*Arhus arhus*) liver oil

6.07 g of a dull reddish yellow oil were obtained from 78 g of liver

This oil was examined in detail

(a) 10 c.c. contained 0.043 g

Blue units	Yellow units	Neutral tints
2.0	0.5	
2.0	0.5	
2.1	0.5	

Mean blue value

2

Carr-Price value

93

(b) 10 c.c. contained 0.086 g of the oil

Blue units	Yellow units	Neutral tints
3.9	1.4	0.1
3.9	1.4	0.1
4.0	1.4	0.1

Mean blue value

3.9

Carr-Price value

90

J, MR

(c) 10 c c contained 0.129 g of the oil

Blue units	Yellow units	Neutral tints
6.9	1.8	0.1
7.0	1.8	0.1
6.9	1.8	0.1

Mean blue value

6.9

Carr-Price value

107

Cod-liver oil (B. C. P. W.) gives a Carr-Price value of about 4.5 to 5.0 (Chakravorty, Mookerjee and Guha, *loc. cit.*)

SUMMARY

The liver oils of *chital*, *shalong*, *kalibans*, *dhain* and *an* have been studied. The Carr-Price values of the oils are of the order of 206, 75, 9, 175 and 93 respectively. The Carr-Price value of samples of commercial cod-liver oil has been found to vary from 4.5 to 5.0.

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INVESTIGATIONS ON THE NUTRITIVE VALUES OF INDIAN FOOD-STUFFS

Part I

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ALTHOUGH a survey of the literature shows that a few of the Indian food-stuffs have been investigated with reference to their nutritive values, a systematic investigation of these does not appear to have been undertaken in this country. A knowledge among other things of the protein values, the vitamin values and of the content of important inorganic constituents of the common food-stuffs is essential both from the standpoint of general nutrition in India and from that of clinicians. The nutritional requirements and habits in India are varied and it seems to us desirable that regional investigations of food-stuffs should be undertaken. It has been found, for example, that different varieties of the Indian mango vary significantly in their vitamin contents (Guha and Chakravorty, 1933a). A rather extensive investigation therefore seems to be necessary.

The present communication describes the first of a series of researches in this line. The protein contents, the vitamin B₁- and B₂-values as well as the contents of calcium, phosphorus and iron—the most nutritionally significant inorganic elements—have been investigated. The potency with reference to other vitamins is also under investigation and will be reported later. The food-stuffs tested are in common use in Bengal.

EXPERIMENTAL

Method of assay of vitamins B₁ and B₂—The biological technique described before (Guha and Chakravorty, 1933a), which is an adaptation from the earlier methods (Guha and Drummond, 1929, Guha, 1931), has been used.

The minimum quantity of food-stuff which would produce a weekly gain in weight of 10 g to 12 g for a period of 2 to 3 weeks in young rats, subsisting on a vitamin B₂-deficient diet, has been taken to contain 1 unit of vitamin B₂ (Guba and Chakravorty, 1933b)* The vitamin B₁ values have been expressed in terms of international units by direct comparison with the international standard, kindly supplied by Colonel Sir Robert McCarrison

Estimation of calcium, phosphorus and iron—The methods described below are typical for all the analyses and are adaptations from standard procedures —

The percentage dry weight of each fresh food-stuff, after washing well with water, was determined in the usual way by drying in the air oven at 100°C–110°C. A weighed quantity of the ash (0.5 g to 1 g), obtained by incineration, was dissolved by digesting with concentrated HCl, evaporated to dryness and extracted with water to remove silica. The filtrate was then analysed for Fe, Ca and P (in terms of P₂O₅) after treatment with dilute HCl and making up to a known volume.

Determination of Fe (colorimetrically)—A standard Fe solution was prepared by dissolving 0.3608 g of ferrous ammonium sulphate in 100 c.c. distilled water. This was treated with 5 c.c. concentrated H₂SO₄, warmed slightly, treated again with potassium permanganate until oxidation was complete and finally made up to 500 c.c. The colorimetric estimations were carried out by comparison with the above standard solution in Nessler's tubes in the usual way (Elvehjem, 1930).

Determination of calcium—A known volume of the ash solution was made just alkaline with ammonia, treated with excess of ammonium acetate (10 c.c. to 15 c.c. of 20 per cent solution) and freed from phosphates by means of dilute ferric chloride. After filtering and washing the precipitate with 1 per cent ammonium acetate solution, the filtrate and washings were treated with 2 g to 3 g ammonium chloride and boiled. One to two grammes of oxalic acid were added to the boiling solution with continual stirring. A few drops of strong ammonia and excess of 3.5 per cent of ammonium oxalate were finally added and stirring continued. The precipitate of calcium oxalate was removed, washed free from chloride with warm 1 per cent ammonium oxalate solution and burnt in a platinum crucible to calcium oxide. The above method was adapted from that of Richards, McCaffrey and Bisbee (1901), who showed that the quantity of magnesium oxalate occluded by calcium oxalate depended on the concentration of undissociated magnesium oxalate and that the quantity could be decreased by addition of ammonium chloride which formed complex compounds with magnesium.

Determination of P₂O₅—The estimations were carried by precipitation with ammonium molybdate by the usual standard technique.

Determination of protein—Nitrogen was estimated by Kjeldahl's method with 1 g of the dried food-stuff. The percentage of protein was calculated by multiplying the figure for nitrogen by 6.25.

The results are given in the table on next page.

* A very similar unit had been proposed earlier by Bourquin and Sherman (1931), which we, unfortunately, overlooked.

TABLE

Bengali names of food stuffs	Botanical names	Vitamin B ₁ (international units per 100 g fresh food)	Vitamin B ₂ (units* per 100 g fresh food)	PERCENTAGE IN THE IDEAL PORTION OF THE IRISH FOODS			
				1 c	Ca	P ₂ O	Protein
<i>Bandha lópi</i> (cabbage)	<i>Brassica oleracea</i>	25	0	0.0017	0.179	0.075	0.85
<i>Dherósh</i>	<i>Hibiscus esculentus</i>	28	2	0.00056	0.12	0.112	1.0
<i>Gma shak</i>	<i>Erythraea roxburghii</i>	25	0	0.0258	0.16	0.0792	2.1
<i>Kānch kalā</i>	A variety of <i>Musa</i> <i>paradisica</i>	4	8	0.004		0.0496	0.93
<i>Kálm shāl</i>	<i>Ipomoea reptans</i>	20	2	0.0264	0.075	0.087	1.7
<i>Lāl shāl</i>	<i>Amaranthus</i> sps	0	10	0.035	0.168	0.105	1.6
<i>Mān Lóchoo</i>	<i>Colocasia Indica</i>	20	20	0.00126	0.019	0.0143	0.75
<i>Pālon shāl</i>	<i>Spinach oleracea</i>	20	20	0.0189	0.103	0.016	2.2
<i>Patól</i>	<i>Trichosanthes dioica</i>	10	20	0.00077	0.054	0.308	1.93
<i>Poon shāl</i>	<i>Bassella cordifolia</i>	40	10	0.00798	0.154	0.434	1.2

* Units as defined by Guha and Chakravorty (1933b)

SUMMARY

Of the common Bengali food-stuffs investigated, *lāl shāk* (*Amarantas sps*), *gimā shāk* (*Erythraea roxburghii*), *kôlmī shāk* (*Ipomœa reptans*) and *pālōng shāk* (*Spinach oleracea*) have been found to be the richest sources of iron. Cabbage, *lāl shāk*, *gimā shāk* and *poorn shāk* (*Bassela cordifolia*) are rich in calcium. *Patôl* (*Trichosanthes dioica*), *poorn shāk* and *dherôsh* (*Hibiscus esculentus*) are important sources of phosphorus. The protein content is highest in *pālōng shāk*, *pātôl*, *gimā shāk* and *kôlmī shāk*. The richest sources of vitamin B₁ are *poorn shāk*, *dherôsh*, *kānch-kalā* (*Musa paradisiaca*) and cabbage. *Patôl*, *pālōng shāk* and *mān-kôchôo* (*Colocasia Indica*) are fairly good sources of vitamin B₂. It should, however, be pointed out that these vegetables cannot compare with cereals in their potency for vitamin B₁ or with mammalian liver in the content of vitamin B₂.

Our thanks are due to Dr S Hedayetullah for his kindness in supplying the botanical names.

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A METHOD OF MAKING SLIDE SMEARS FROM FEMALE
ANOPHELES, FOR EXAMINATION FOR SPORO-
ZOITES OF MALARIA PARASITES, AND
OF PRESERVING THE MOSQUITOES
FOR REFERENCE

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THE following method of making preparations from female *Anopheles* mosquitoes, for later examination for sporozoites of malaria parasites is useful. This is especially the case when making rapid tours, and when ordinary laboratory facilities, or apparatus, are not available for more complete dissections. The mosquitoes are also preserved for identification, future reference, and more detailed study. This is important when a survey of a new area is being made. It also permits of reference being made to any of the specimens at a later date, should questions arise in connection with other researches or classification of species.

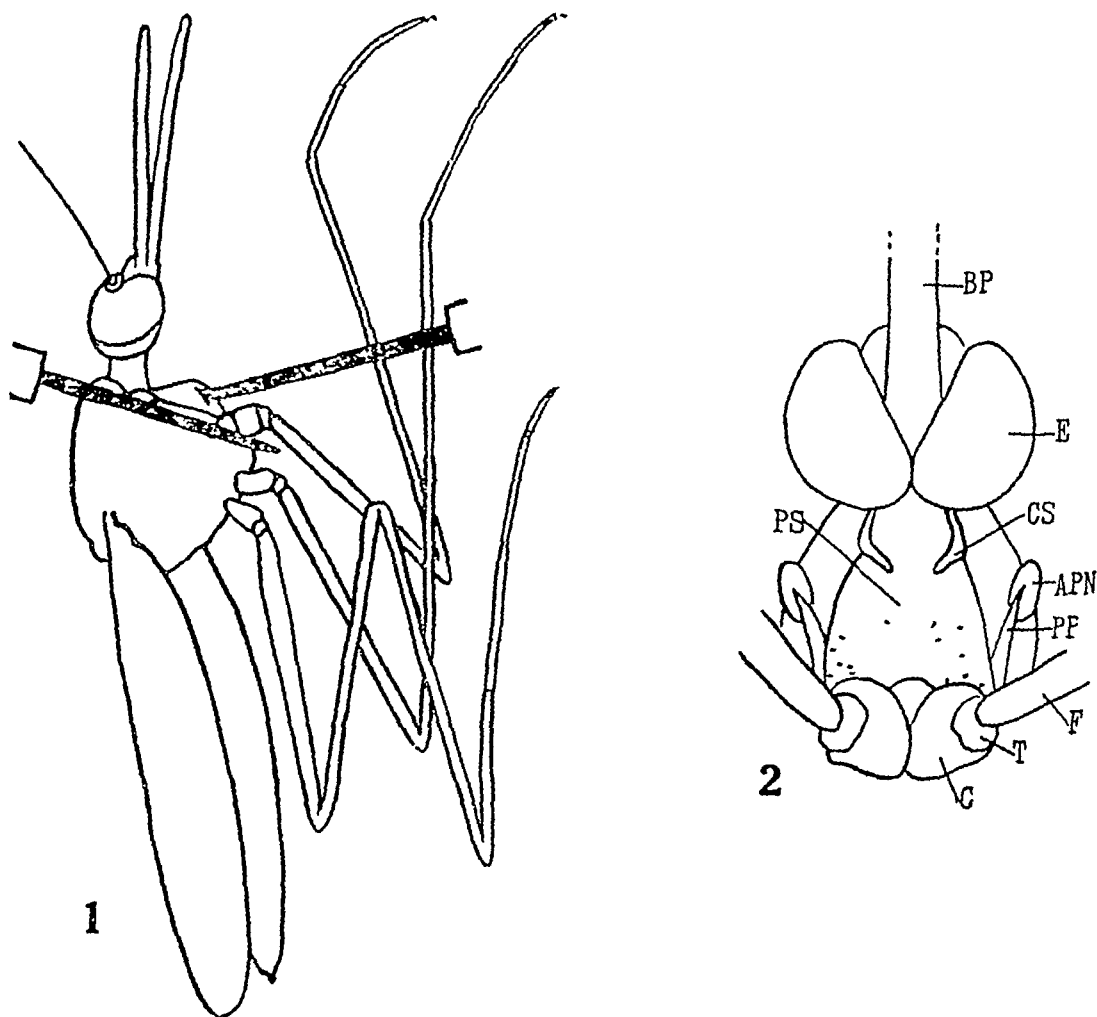
The method is a combination of one, used successfully by Laboratory Assistants of the Malaria Survey of India, for dissection of the salivary glands of mosquitoes, of the Sergeants' technique for detecting sporozoites in the body-fluid of these insects (Sergeant and Sergeant, 1928), and of a method of making slide smears of the salivary glands.

The apparatus required is as follows. A supply of test-tubes with wool-plugs, a supply of microscopical slides, two needles, or pins (preferably mounted in handles), some staged-tubes, a supply of small double-pointed entomological pins and a pair of entomological forceps. A chloroform tube*, or a supply of chloroform, is also useful. The slides may be prepared previously by marking off three spaces by drawing transverse lines with a diamond, about one inch apart, and numbering these consecutively.

The mosquitoes are caught in test-tubes. When a preparation is to be made, kill the insect with chloroform, or by blowing tobacco-smoke into the tube. When

* See *Health Bulletin* No 14—*Malaria Bureau* No 6 'How to do a Malaria Survey' 2nd Ed., 1931, p 34

dead, turn the specimen out on to a slide. Arrange the whole mosquito on a numbered square, on its left side, with the head pointing away from you. Do not add any saline, or other fluid, as this is not required. Do not remove the wings or legs, and endeavour to carry out the following manipulations with as little damage



TEXT FIGURES

- Fig 1 Outline drawing of *Anopheles* female (legs of one side only shown) to illustrate method of dissecting. Left hand needle pressed on to anterior part of thorax, and right hand needle piercing 'prosternum' which is bulged out by pressure of the other needle. Point of right hand needle is shown inserted exactly in the region of the salivary gland of one side.
- „ 2 Underside of head and prothorax of *Anopheles* female (drawn from freshly killed unmounted specimen). The shaded parts indicate the region of the salivary glands within the thorax. Lettering —BP, base of proboscis, E, eye, CS, cervical sclerite, APN, anterior pronotal lobe (prothoracic lobe), PP, propleura, C, coxa of front leg, T, trochanter, F, base of femur, PS, 'prosternum'.

as possible to the scaling, etc. Take a needle in the left hand and place the shaft of this flat across the front of the thorax as shown in Fig 1. Press down with this so as to cause the thin chitin of the front of the thorax below the neck ('prosternum')

to bulge out considerably (Fig 1) The salivary glands lie just within this thin sheet of chitin (see Fig 2) With a needle held in the right hand cut into this part (Fig 1) Draw out, and cut off, the tissues which will protrude, and by continued pressure with the left-hand needle at the same time, squeeze out some of the body-fluid on to the slide Endeavour to avoid severing the neck, or detaching the front legs Tip the mosquito on to a piece of paper, and with a needle rapidly* mash up and make a small circular smear, on the numbered square, of the tissues and fluid left on the slide Dry this by waving the slide in the air It should be noted that the dissection and smear are made *without* any admixture of saline When opportunity occurs the smear should be fixed with methyl-alcohol or absolute alcohol, and the slides stored for staining and examination, which may be done at any convenient time later

Pin the mosquito and mount it in a staged-tube, marking on the stage the number corresponding to the smear on the slide Observations on the locality, the nature of the place where the specimen was collected, the date, and any other details considered worth recording, should be entered in a note book at the time

Before making another preparation clean the needles thoroughly

When required for examination the slides should be stained with Giemsa stain and search made for the presence of sporozoites It is most probable that these will be found in all cases where the mosquitoes contain infection of the glands This is so because it is very probable that one or both of the salivary glands will have been extracted with the tissue and be included in the smear, as these protrude as soon as the 'prothorax' is cut into†

It has been shown by Mayer (1921) and by Muhlens (1921) that after rupture of a mature oocyst on the gut-wall of the mosquito, sporozoites are distributed all over the body and can be demonstrated in the body-fluid and even in that of the appendages Strickland and Roy (1931) state that they found sporozoites in the body-fluid of all mosquitoes examined in which the glands showed infection

More recently Strickland, Chowdhury and Chaudhuri (1933) have examined large numbers of *Anopheles* in the Bengal Terai by the Sergeants' method, with apparently very successful results

The advantages of the technique described above may be summarized as follows —

(1) The method combines, in the large majority of preparations, the advantages of dissection of the salivary glands, with those of Sergeants' method for detecting the presence of sporozoites in the body-fluid of mosquitoes

* This is important in very dry climates

† It has been found by some of the technicians employed by the Malaria Survey of India that with a little practice, dissections of the salivary glands can be made, if desired by this method, both glands being obtained in practically 100 per cent of cases and without any material damage to the mosquito Under these conditions the glands can be transferred to a small drop of saline and examined by the ordinary method in the fresh condition It is also possible, by turning the mosquito on its back, with the head still pointing away from you, to extract the mid gut and viscera The specimen is held with the left hand needle lightly pressed between the legs, and the gut withdrawn with the right-hand needle by pulling on the terminal segments, after these have been partially severed by nicking the chitin in the ordinary way The gut is then drawn along the slide into a small drop of saline, and the mosquito removed, pinned, and mounted So long as the mosquito is kept free from the saline the operations can be carried out with little damage to the wings, palpi, or legs, and the specimen is preserved in a sufficiently good condition for identification

(2) The insect host is preserved for identification and for reference in the future

(3) The method can be carried out rapidly in the field where laboratory facilities, and sufficient time, for carrying out dissections in the ordinary way, are not available

(4) The preparations can be made by an assistant not necessarily trained in the detection of sporozoites, nor in the identification of mosquitoes

(5) A microscope is not necessary for making the preparations

The only disadvantage is that no information is obtained regarding the presence or otherwise of oocysts on the gut-wall. Although Swellengrebel and de Buck (1931) have maintained that 'in *A. maculipennis* the incidence of intestinal infection allows of a more accurate estimate of the actual number of sporozoite carriers than does the incidence of salivary infection', it should be remembered that these conclusions were based on investigations made under the favourable and sheltered conditions of experimental work, and not under the more dangerous conditions ruling in nature. Under the latter conditions James (1926) estimates that 'the only mosquitoes which succeed in transmitting malaria are those rare individuals who happen to pass their life in conditions which resemble very closely those which we have found to be essential for the successful transmission of the disease in experimental work', i.e., a sheltered life. It seems therefore likely that a proportion of mosquitoes found in nature showing intestinal infection would, under natural conditions, fail to survive for a sufficient time for the glands to become infected, or certain other factors may prevent the appearance of sporozoites in this position. Conclusions based on the incidence of gut-infections only may therefore not give an accurate estimate of the transmitting power of a particular species in nature at any given time, as shown by Iyengar (1931). On the other hand the presence of sporozoites in smears obtained by the method given above would afford conclusive evidence regarding those species which are in the infective stage. This gives some measure of the danger at the actual time of the examinations.

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THE LONGEVITY OF FEMALES OF *CULEX FATIGANS* UNDER EXPERIMENTAL CONDITIONS, AND THE DURATION OF MALARIAL INFECTIONS IN THESE INSECTS

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DURING the course of other experiments the following data were collected about the longevity of, and the duration of malarial infection in, *Culex fatigans*, the common domestic culicine mosquito of India. This investigation was carried out at the Ross Field Experimental Station for Malaria, Karnal (Punjab), between 22nd October, 1932 and 20th May, 1933.

TECHNIQUE OF EXPERIMENTS

A large number of pupæ of *C. fatigans* were collected from natural breeding places of these insects. The adults which emerged from these were kept for 12 hours without food or drink in a muslin cage about 18 inches square. At dusk on 22nd October an open wire cage, containing a specimen of the common house sparrow (*Passer domesticus*) showing a heavy natural infection with *Plasmodium* sp (*Proteosoma*), was placed in the muslin cage with the mosquitoes. The cage was covered with a dark cloth and left overnight. Next morning blood was visible in the abdomens of 110 mosquitoes. These were collected from the cage and transferred to the small lamp-chimneys described below. Five insects were placed in each chimney.

These chimneys are of the type commonly used with small 'hurricane' oil lamps, and are about 4 inches long by $2\frac{1}{2}$ inches in greatest diameter at the globular portion. The end openings, about $1\frac{1}{2}$ inches to $1\frac{3}{4}$ inches in diameter, were each closed with a piece of mosquito netting, which was held in position by a tape or a rubber-band. The chimneys were placed upright in a wooden

tray, on the bottom of which several layers of damp lint had been placed. The upper end of each was partly covered with a strip of damp lint, beneath which were placed a few raisins in apposition with the netting over the chimney. The lint both above and below the chimneys was moistened with water once every 24 hours. Previous experience had shown us that a growth of mould was liable to occur on the lint, netting and raisins, and that this had a marked effect in increasing the mortality among the insects*. It was therefore necessary to change these as soon as any sign of mould was detected.

The original feed was the only blood meal given. Although the insects were given opportunities of feeding on a sparrow on 7 occasions during the 15th week, none fed. The mosquitoes therefore subsisted upon the raisins and water during the whole period after the original feed had been digested.

The chimneys were kept in an ordinary room until the end of April. During this time a careful record was kept of the maximum and minimum temperatures in the room and also of the relative humidity at 8 a.m. and 4 p.m. (*vide* Tables I and II). No artificial heat was used during the winter. When necessary the floor was sprinkled with water once or twice daily to ensure a high relative humidity at all times†.

At the 13th week of the second period, as there was a marked fall in the relative humidity of the room and a rise in temperature (*vide* Table II), the remaining mosquitoes (5 in all) were transferred to a room which was artificially cooled and humidified‡. The records given during the 14th and 15th weeks of the second period refer to this room.

* This increased mortality due to infestation of the insects with bacteria and moulds has also been noted by other workers. Sections of mosquitoes dying under these conditions, show the gut blocked with such organisms, which are sometimes seen invading the tissues of the host. It was impossible to say whether the invasion was purely post mortem or not.

† This is the routine method started by Dr. Bruce Mayne and used since in the Malaria Survey of India, when Culex infected with bird malaria are required for any purpose. It is found to work well. The temperature conditions indoors at Karnal are usually suitable for the development of the sporogony cycle of the bird *Plasmodium*. On the other hand, the relative humidity is frequently unfavourable for the survival of the insects over a prolonged period in captivity. The necessity for a moist atmosphere in such experiments seems to have been first noticed by Nuttall and Shipley (1902). Gill (1921) found that with *C. fatigans* the lower limit of relative humidity, compatible with the life of these insects up to 5 days (at a mean temperature of 27°C), was a mean of 48 per cent, a relative humidity of 60–80 per cent at the same temperature was favourable to life up to 10 days, while a relative humidity over 80 per cent appeared to be even more favourable. Mayne (1930) arrived at somewhat similar conclusions in his work with the same species of insect. For this reason in our routine work a high relative humidity is maintained by means of moist lint over the lamp chimneys and by watering the floor of the room where the insects are kept. The relative humidities shown in Tables I and II indicate the effects of the latter measure only. The humidity inside the chimneys, as the result of the moist lint, must have been about 80 per cent or over on most days.

‡ The system used in this room was devised by Dr. G. Macdonald of the Malaria Survey of India, on the suggestion of, and in consultation with, Major General Sir John Megaw, C.I.F., Director General, Indian Medical Service. This was a successful attempt to determine whether a cheap and effective method could be devised to maintain a more comfortable atmosphere in a room during the hot dry months in the Punjab, if electric power were available.

One doorway of the room was closed with a 'khas khas' (loose root fibre) screen, down which a continuous flow of water could be maintained. The air was drawn into the room through this moist screen by an ordinary electrical table-fan placed in the room close to the screen and a few feet from the floor. The hot air was driven out by a similar fan placed at an opening near the roof. The doors and windows were kept shut, as far as possible, while the system was in operation.

A somewhat similar arrangement with a hand driven fan had previously been in use.

RESULTS OF EXPERIMENTS ON LONGEVITY

It has been found convenient to divide the 210 days, during which the experiments lasted, into two periods of 15 weeks each. The first from 22nd October, 1932 till 4th February, 1933, is roughly the cold season of the year at Karnal, while from 5th February till 20th May, 1933, was a warmer period (*vide* Tables I and II)

First period of the experiment

No insects died during the first two weeks and of the 110 mosquitoes with which the experiment started, 40 were alive at the end of 15 weeks. The deaths of 59 insects were attributable to natural causes, while 11 were killed to determine the presence of malarial infection.

The maximum room temperature during this period varied between 56°F and 78°F (average 64.4°F) with minimum temperatures of 53°F to 73°F (average 60.7°F). The mean relative humidity in the room during this period ranged from 51 to 77 per cent (average 66.9 per cent) at 8 a.m., and from 44 to 74 per cent (average 60.7 per cent) at 4 p.m. Under these external conditions it seems highly improbable that the relative humidity inside the lamp-chimneys ever dropped below 60 per cent. It was probably at 80 per cent or over during most of the period.

Summary of results obtained during the first period

Ninety-nine specimens of *C. fatigans* (♀♀) were given one meal of avian blood and placed under experimental conditions during which they received only raisins and water. During the 15 weeks of this part of the experiment, the temperature ranged between a minimum of 53°F and a maximum of 78°F, with an average of about 62.5°F. The relative humidity in the containers was high, probably never below at least 60 per cent and often 80 per cent or over. Under these conditions 40 per cent of the insects survived for 105 days.

Second period of the experiment

During the second period of 15 weeks, of 40 mosquitoes which were alive at the commencement, only 1 was alive at the end. Seventeen insects died of natural causes, 13 were lost and 11 dissected to determine the presence of malarial infection.

The maximum room temperature during this period varied between 59°F and 81°F (average 72°F) with minimum temperatures between 58°F and 78°F (average 69°F). The mean relative humidity in the room ranged from 53 to 77 per cent (average 67.5 per cent) at 8 a.m. and from 36 to 65 (average 52.7 per cent) at 4 p.m.

Summary of results obtained during the second period

The observation of the 40 mosquitoes which had survived for 15 weeks was continued for a further period. During this period the room temperature was higher (*vide* Table II). This varied between a minimum of 58°F and a maximum of 81°F, with an average of 70.5°F. The average relative humidity was lower than during the previous period, but probably never fell as low as 50 per cent in the

chimneys Under these conditions 5 mosquitoes survived 84 more days, i e., until transferred to the cooler room Of these 1 was killed for dissection on 91st day and the survivor was killed on 105th day These results taken in conjunction with those of the previous period make a total of 189 days of survival for 5 insects, at least 196 days for one insect and at least 210 for another

SOME RESULTS RECORDED BY OTHER WORKERS

Stephens and Christophers (1908) say that 'in nature, Anophelines certainly remain alive in huts for one or two months and possibly longer' Howard, Dyar and Knab (1912) report that Knab in 1903 made observations which suggested that certain species of *Aedes* lived in nature at least 3 months during the summer in western Massachusetts Herms (1915), basing his opinion on the results of measures taken against mosquitoes, estimates the duration of life of an adult female insect as 30-40 days

Stephens and Christophers (1908) note that under experimental conditions, if suitably housed and constantly fed, mosquitoes may be kept alive for days, weeks and even months Hegh (1921) states that female mosquitoes can be kept alive on ripe bananas or blood for 2 months, but this period is more than the average, generally all die in 2-3 weeks Howard, Dyar and Knab (1912) record that the females of *Aedes aegypti* have been kept alive in captivity for 154 days Mayne (1922) kept a specimen of *C territans* alive for 265 days on a diet of dates and water

Workers like Gill (1921) and Mayne (1930) have kept *C fatigans* alive for several weeks in captivity, under suitable conditions of humidity and temperature Mayne (1930) working with this species found that the minimum range of relative humidity for the insect was 43-45 per cent even at a temperature above 80°F Of a batch of 47 females of *C fatigans* kept at 80 per cent relative humidity and 85°F, 27 were still alive at the end of 34 days, the majority of the others having been killed for dissection

Many workers have experimented with the longevity of Anophelines in captivity Nuttall and Shipley (1902) state that Grassi kept Anophelines alive for a month under laboratory conditions Howard could only keep *A maculipennis* alive for 8 days during the summer, but in the autumn they lived for 50-60 days in confinement, these being insects which would probably have hibernated

Mayne (1922) investigated the longevity of *A punctipennis*, *A crucians* and *A quadrimaculatus* under the same conditions The insects were confined in lamp-chimneys at a range of temperature from 48°F to 76°F After 1-3 preliminary feeds on blood, a diet of dates and water only was given Specimens of the first species survived 100 days, of the second 65 days and of the last 73 days under these conditions In another experiment a specimen of *A punctipennis* was kept alive for 231 days

James (1926) records that, of a batch of 300 specimens of *A maculipennis* caught in nature and fed on an infected person, 2 were alive after 96 days and 1 after 103 days These insects had lived intermittently for 3 weeks at 73°F-75°F and 2½ months at about 40°F-43°F During this time they had had 40 opportunities of feeding on blood The numbers surviving this period would probably have been much greater, because many were killed for dissection and the insects were

exposed to the risks of transport and experimental manipulations in connection with the transmission of malaria to man on 40 occasions James (1926) also reports that there was a 50 per cent mortality among his specimens of *A. maculipennis* every 7-10 days, when kept under artificial conditions at about 72°F-75°F At about 79°F, the mortality was still higher

Mayne (1930) found that the minimal humidity requirements for *A. fuliginosus* were 38-40 per cent, for *A. subpictus* and *A. stephensi* 55-58 per cent and for *A. culicifacies* 57-62 per cent At a relative humidity of from 55 to 58 per cent with temperatures of from 80°F to 88°F *A. culicifacies* survived in captivity from 7 to 21 days, *A. subpictus* from 5 to 39 days and *A. stephensi* from 7 to 32 days

DISCUSSION OF RESULTS

James (1926) considers that mosquitoes under experimental conditions lead a sheltered life They are protected against wind and rain, against unfavourable changes of temperature, and their food is available without risk To these may be added protection from two of the great risks under many tropical conditions (a) protection from natural enemies, especially from ants, which attack sluggish insects, and (b) protection from unfavourable conditions of low humidity James (1926) thinks that with *A. maculipennis*, if the temperature in nature be for 10 days or more about 75°F, only one mosquito out of five which have had infective meals, survives long enough to infect another person He also associates the shorter life of this species in the earlier summer with the greater reproductive activity of the insect at this time, as compared with the autumn

Boyd (1930) states that 'the adaptation of the schizogonous (? sporogonous) cycle of the malaria parasite to the anopheline mosquito indicates that the probable expectancy of life of the summer female may be two weeks' This suggestion may also include *C. fatigans*, the common host of the bird *Plasmodium* in India The fact that all other insects survived two weeks under experimental conditions, may indicate that this is about the usual period of survival when the humidity conditions are favourable

The various factors which have been suggested by different workers as having a marked influence on the longevity of the mosquito, both in nature and captivity, must be considered

In discussing the survival of *C. fatigans* under artificial as compared with natural environments, one must remember that this is a house-haunting mosquito It probably remains indoors when meteorological conditions are unfavourable Its domestic propensities make available an abundant supply of animal food When the humidity conditions outside are so low as to be unfavourable, the insects are free to find more suitable conditions indoors and therefore haunt bathrooms and the vicinity of any stored water Local conditions of relative high humidity are not uncommon in human habitations in India The temperature in the plains of the Punjab and most parts of India, never falls low enough during a sufficiently long period for hibernation to take place

As stated by James (1926), the conditions of life in captivity were much more favourable for his mosquitoes in many respects than in nature It is therefore necessary to consider in how far the experimental conditions affected the insects in our investigations

The conditions favourable to survival may be summarized as follows —

- (a) *Absence of natural enemies* — Under natural conditions in India there is no doubt that ants and other enemies destroy large numbers of mosquitoes, more especially those which are sluggish for any reason. Our insects were protected from this danger.
- (b) *Presence of constant and favourable conditions of humidity and temperature* — In the plains of the Punjab, where, for considerable periods of the hot weather, the outdoor humidity conditions may be unfavourable, our insects were protected from these adverse circumstances. However, as mentioned previously, in nature the insect has often many opportunities of escaping such conditions. In many other parts of India the humidity conditions for this insect are very favourable during most of the year.
- (c) *Protection from wind and rain* — As mentioned previously such protection may be available in nature.
- (d) *Absence of a high degree of reproductive activity* — As our insects were bred in captivity, probably most of them were unfertilized and the risks of egg maturation and laying were avoided.
- (e) *Easily available food supply* — Except for their first meal, our insects did not get any of their natural blood diet. Whether such a diet would prolong life we do not know, but some of the work of James (1926) is suggestive. The food supplied in our work was liable to increase the mortality from bacterial and fungus contamination.

The conditions unfavourable to survival may be summarized as follows —

- (a) *Bacterial and fungus infection from food*
- (b) *Absence of opportunity of seeking the most favourable natural conditions of temperature and humidity*
- (c) *Absence of normal food (?)*
- (d) *Injuries under experimental conditions*
- (e) *Absence of opportunity for changing diet (?)*

CONCLUSIONS

It is impossible from these experiments to say what is the normal length of life of females of *C fatigans* in nature. If one compares the favourable and unfavourable conditions present in captivity, it seems probable that the majority of these insects live at least a month in nature, and probably for a much longer period in localities where the humidity conditions are favourable. As this insect is a common carrier of filariasis in many parts of India, further experiments on its longevity seem indicated in relation to the study of this important disease.

RESULTS OF EXPERIMENTS UPON THE DURATION OF INFECTION IN *Culex fatigans*

During the course of the experiments summarized above, 21 mosquitoes were dissected to determine the presence of malarial infection arising from the blood meal given at the commencement of the investigation. Of these mosquitoes a total of 17, or 81 per cent, showed infections, either with oocysts in the gut or sporozoites

in the salivary glands. Gut infections were found in 6 insects and infection of the salivary glands in 21 (*vide* Table III)

During the 15th week, i.e., at the end of the first period mentioned above, dissection showed that a very large proportion of the insects still had numerous motile sporozoites in the salivary glands. These appeared to be perfectly normal and were actively motile. As a further test of the viability of these organisms, the insects were given an opportunity of feeding upon a normal sparrow. Unfortunately, although they had opportunities on 7 different occasions, none fed. This may have been due to (a) the low temperatures prevailing at the time, or (b) because the insects, having become accustomed to a fruit diet, had lost their avidity for blood, or (c) because the insects were enfeebled by long captivity (which does not seem likely, when one considers that at least 5 of them lived for a further period of 84 days), or (d) because the absence of suitable conditions for fertilization and oviposition affected the insects unfavourably.

It seems most probable that the main cause of the failure to feed was the adverse conditions of temperature.

When the attempts to induce the insects to feed had failed, it was decided to try whether the infection could be transmitted by the injection of sporozoites from the glands into normal sparrows. The glands were dissected out in normal saline solution, and the presence and motility of the sporozoites determined under the microscope. The infected glands were then emulsified in saline solution and the resultant mixture drawn up into a pipette with a very fine point. With this the sporozoites were injected directly into the wing vein of the experimental birds. The whole procedure did not take more than 5 minutes from beginning to end.

The results of these experiments are given in Table IV. From this table it can be seen that no infection was produced in one bird by the intravenous injection of sporozoites derived from a total of 30 lobes of infected salivary glands. This total amount was injected at four different times from mosquitoes showing infection from 16th to 19th weeks. In all instances the sporozoites were actively motile and appeared to be normal.

To the second sparrow, the sporozoites from 6 lobes of infected glands were injected on each of two occasions, in the 24th and 28th weeks respectively. On the first occasion the sporozoites were motile and on the second they were non-motile. Here again no infection developed.

From Table III it will be seen that 8 out of 10 mosquitoes dissected during the second period showed sporozoites in the salivary glands. Actively motile sporozoites were found as late as the 166th day after the primary feed, while non-motile sporozoites were found at 194th and 210th days.

DISCUSSION

Several workers have investigated the duration of sporozoites in the salivary glands of infected mosquitoes. Ronbaud (1918) fed 5 bred specimens of *A. maculipennis* on a patient infected with *P. falciparum*. Three of these insects showed a heavy oocyst infection of the gut 7 to 12 days later, and a fourth had its salivary glands crammed with sporozoites when examined on 25th day. The remaining mosquito was kept in the laboratory at temperatures varying from 39°F to 74°F for 2½ months, and fed on syrup. During the next 23 days it was allowed to feed

on various animals on 4 occasions. When this insect was allowed to bite a man 106 days after the original feed, no infection was produced. It was killed and dissected at the end of 125 days, when only a few degenerate sporozoites were found in one gland. Roubaud concludes from this that an infected Anopheline may purge itself of sporozoites in the course of a few bites and that any sporozoites not so discharged gradually degenerate.

Mayne (1922) reports that he has seen a few scattered sporozoites in the glands of a specimen of *A. punctipennis* fed 158 days previously on a patient harbouring *P. vivax*. In a later series of experiments Mayne (1922) tried to infect a patient with a batch of *A. punctipennis* which had fed on a crescent carrier 74 days before and been kept at temperatures between 45°F and 75°F. No infection was produced. On dissection of these insects some time afterwards, 'nucleated, sluggishly motile bodies, indistinguishable from sporozoites', were found in the salivary glands of two. These mosquitoes had therefore retained living sporozoites of *P. falciparum* in their glands for 83 to 92 days after feeding. A similar test was made with another batch of the same species of mosquito also fed on a case of malignant tertian malaria. These insects conveyed infection successfully after 55 days, but failed at 67th day, although very active sporozoites were found on 68th day. In another instance under similar conditions, a mosquito, which showed 'viable sporozoites' on 70th day, had failed to infect a patient on 61st day. Another specimen, which failed to transmit infection on 66th day, harboured living sporozoites on 71st day. In Mayne's study '95 days was the longest time that apparently dead sporozoites were found in the salivary glands of specimens of *A. punctipennis* infected with *P. falciparum*, and in those infected with *P. vivax*, 105 days was the maximum period'.

James (1926), in the experiments summarized previously found that *A. maculipennis* could convey infection by bite as late as 95 days after one infective feed on a patient with benign tertian malaria. The salivary glands of the specimen which transmitted this infection were found to contain on 102nd day 'numerous sporozoites which were actively motile and showed no signs of abnormality'. The mosquito's stomach was 'full of uncoagulated red blood but the presence of zygotes (oocysts) was determined'.

In evaluating this experiment in relation to those with *C. fatigans*, it must be remembered that the Anophelines used had spent at least two-thirds of the time at temperatures ranging between 40°F and 43°F. These low temperatures must have markedly slowed down the development of the sporogony cycle of the malarial parasites in the insects, as is shown by the presence of oocysts at a late period. The gradual maturation of these oocysts, when conditions were favourable, must have resulted in a continual replenishment of the store of sporozoites in the salivary glands. Under these conditions the age of the sporozoites at the end of the experiment cannot be determined, but was almost certainly very much less than in the insects reported by Roubaud (1918), by Mayne (1922) and in our work.

James (1927) also reports that a batch of *A. maculipennis* fed on patients infected with *P. vivax* transmitted the infection to 54 new patients during a period of 10 weeks. During the last 2 weeks sporozoites became scanty but were replenished by refeeding on an infected person. After this they succeeded in infecting 3 other persons. The conditions of this experiment were very similar to those previously recorded by James (1926).

CONCLUSIONS

The results of our experiments and those of other workers recorded above suggest that —

(a) After a single infective feed a mosquito may transmit the malarial parasite for a period as long as 8 weeks, when kept at comparatively high temperatures for most of the time

(b) If the infected mosquitoes are kept for the majority of their lives at comparatively low temperatures sufficient to inhibit or retard the growth of the parasite the salivary glands may be continually replenished with fresh sporozoites derived from oocysts maturing during periods of more suitable temperature. Under such conditions infections may be transmitted as late as 102 days after the primary infective feed

(c) The sequence of events appears to be different when, after an infective feed, mosquitoes are kept under temperature conditions favourable to a comparatively rapid maturation of all the oocysts and an invasion of the salivary glands with sporozoites. Under such conditions there seems to be a tendency for the sporozoites to lose their infecting power after about 8 or 9 weeks. Even although they are found to be actively motile and show no detectable abnormality, they may not be infective. In *C. fatigans* active, but apparently non-infective, sporozoites are detectable as late as 166 days after a single infective feed, and non-motile sporozoites as late as 210 days

(d) If a mosquito be given periodical feeds of infective blood the duration of infectivity in the insect would appear to be limited only by its span of life

(e) In the absence of replenishment of the glands, biting appears to deplete the stock of sporozoites

Our thanks are due to Laboratory-Assistant Ambrose David for the care taken, and the technical skill displayed, during the course of these investigations

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TABLE I

Colden period from 22nd October, 1932 to 4th February, 1933 (15 weeks)

Number of insects receiving infected feed	Average deaths per week										4 or 4 per cent*		Total insects dying of natural causes						
													Total insects killed						
											Total insects surviving					59	11	40	
	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week	9th week	10th week	11th week	12th week	13th week	14th week	15th week	Average			
Gland infection	—	0	—	—	+	+	—	—	—	—	—	—	—	—	+				
Gut infection	—	+	—	—	+	0	—	—	—	—	—	—	—	—	0				
Average weekly relative humidity 8 a m (per cent)	72	62	61	51	53	67	59	71	64	76	74	77	67	76	73	66.9			
Average weekly relative humidity 4 p m (per cent)	61	59	53	44	51	58	48	51	56	67	74	74	67	74	73	60.7			
Average weekly maximum room temperature (°F)	78	77	74	72	70	66	66	63	60	61	56	57	55	56	56	64.1			
Average weekly minimum room temperature (°F)	73	72	69	67	66	62	62	60	53	57	53	54	54	55	54	60.8			

* Calculated on the numbers dying from natural causes and excluding those killed for dissection

TABLE II

Warmer period from 4th February, 1933 to 20th May, 1933 (15 weeks)

Number of insects surviving first period	Average deaths per week										Total insects dying of natural causes					
	1 or 6 per cent*										Total insects lost during period	Total insects killed for dissection	Total surviving but killed at end			
40	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week	9th week	10th week	11th week	12th week	13th week	14th week	15th week	Average
Gland infection	+	+	+	+	—	—	—	—	+	—	—	—	+	—	+	
Gut infection	0	0	0	0	—	—	—	—	0	—	—	—	0	—	0	
Average weekly relative humidity 9 a.m. (per cent)	71	70	69	74	74	77	66	70	63	61	56	57	53	74	77	67.5
Average weekly relative humidity 4 p.m. (per cent)	60	60	54	61	61	61	41	55	49	41	45	42	36	59	65	52.7
Average weekly maximum room temperature (°F)	59	62	67	65	63	71	72	75	73	73	78	80	91	80	78	72.1
Average weekly minimum room temperature (°F)	58	61	62	59	61	63	68	71	72	70	75	75	78	78	76	68.9

* Calculated on the number dying from natural causes and excluding those lost or killed for dissection

† Showing non motile sporozoites

TABLE III

RESULTS OF DISSECTION OF MOSQUITOES

Weeks	GUTS		PAIRS OF GLANDS		Total mosquitoes examined	Total mosquitoes infected
	Number examined	Number positive	Number examined	Number positive		
2	5	3	5	0	5	3
5	2	1	2	2	2	2
6	1	0	1	1	1	1
14	2	2	2	1	2	2
15	1	0	1	1	1	1
16	2	0	2	2	2	2
17	1	0	1	1	1	1
18	3	0	3	1	1	1
19	1	0	1	1	1	1
24	1	0	1	1	1	1
28	1	0	1	1*	1	1
30	1	0	1	1*	1	1
TOTALS	21	6	21	13	21	17

* Non motile sporozoites

TABLE IV

RESULTS OF INTRAVENOUS INJECTIONS OF SPOROZOITES

Week of injection	Bird number	Number of glands used	Condition of sporozoites	Results of blood examinations of sparrow
16	1 F	12 lobes	Motile	17th week Negative
17	"	6 "	"	18th " "
18	"	6 "	"	19th " "
19	"	6 "	"	20th " "
				(The bird died with no evidence of malarial infection)
24	151 M	6 "	"	25th week Negative
				26th " "
				28th " "
28	"	6 "	Non motile	29th " "
				30th " Negative and also 5 subsequent examinations

NOTICE.

Courses of Instruction in Malariaology under the Auspices of the Health Committee of the League of Nations

THE Health Committee of the League of Nations has arranged for courses of Instruction in Malariaology under the conditions prevalent in Far Eastern countries for the benefit of malariaologists taking up duty in Eastern countries and for those engaged in work in the East who are unable to attend malaria courses in Europe

Courses will be organized annually for theoretical and practical training in Singapore, and field study also arranged for in Malaya and Java The theoretical training will last approximately thirty days, the first course commencing on April 30th, 1934 The practical training will occupy a similar period

The course will be open to any medical practitioner, the fee for the theoretical study being seventy-five Straits dollars Further information may be obtained from the Director of the Eastern Bureau, Health Organization of the League of Nations, Singapore

The Syllabus for the course is appended

Editor

SUMMARY OF SYLLABUS

MALARIA COURSE

I Haematology.

Preparation of thin and thick blood films and preparations for the study of fresh blood
Methods of staining Normal and abnormal blood cells Differential leucocyte counts
Enumeration of red and white cells Estimation of hæmoglobin Anæmias

II Protozoology

- (a) General consideration of Protozoa with particular reference to the Sporozoa—*Plasmodium*, *Hæmoproteus*, *Leucocytozoon*, *Babesia*, *Theileria*, *Hæmogregarina*
- (b) *Plasmodium* Historical, bird malaria and monkey malaria General description and life history of the malaria parasite Development of the parasite in the mosquito Detailed descriptions of *P. vivax*, *P. malariae*, and *P. falciparum* Other species described in man and allied species Mixed infections, relation of parasite cycle to febrile symptoms Cultivation of malaria parasite Difficulties and fallacies in examination of blood

Notice

III. Symptomatology and Pathology.

Ætiology, types of malarial fever, mechanism of febrile attack, onset, course and termination of ordinary cases Clinical types and other diseases simulated, racial variations, pernicious and chronic malaria, provocation of attacks, diagnosis, aids to clinical diagnosis, prognosis Induced malaria, delayed manifestations, complications and sequelæ

Blackwater fever

Ætiology Evolution and symptoms, prognosis, differential diagnosis

Treatment—General treatment

Pharmacology and toxicology of drugs used in treatment, mass and routine treatment, treatment of Blackwater fever and severe and fulminating cases treatment of special symptoms, after treatment and results

Pathology of blood and urine, malarial pigmentation, lesions in organs and their origin

Pathology of Blackwater fever

IV. Entomology.

Insects and their classification Diptera Nematocera Nomenclature, Genus, species, and categories below the species Mosquitoes—general characters, classification and affinities Morphology of adult, larva and other stages in the life history Ecology of adults and larvæ Control Natural enemies and parasites Biological control Prophylaxis Malaria parasite in the mosquito Natural and laboratory infections Susceptibility Distribution of Malaria carriers Epidemiology in relation to mosquito and its parasites Meteorological factors Habits, breeding places and control of principal carriers in the world Technique of catching, handling, rearing, preserving and dissecting mosquitoes Mosquito surveys Determination of mosquitoes in all stages Use of keys and original descriptions in the study of the important carriers in the East

V. Epidemiology.

Endemic, epidemic and pandemic malaria The measurement of malaria Factors influencing prevalence of malaria, e.g. standard of living, meteorology, movements of population, degree of anophelism and seasonal incidence of mosquitoes, susceptibility and immunity Spontaneous disappearance of malaria Anophelism without malaria Topographical

Control (Also see under Entomology)

Destruction of adults Destruction of larvæ species control, and permanent and temporary measures Relation of permanent measures to civil engineering Legislation

Prophylaxis—personal Segregation of infected persons Mass distribution of quinine, plasmoquine, etc Screening Zooprophylaxis Bonification Surveys and their interpretation Organization of Malaria Services

SOME SOURCES OF VITAMIN C IN INDIA

THE ANTISCORBUTIC VALUE OF VARIOUS KINDS OF
SPROUTED MUNG (*PHASEOLUS MUNG*) AND
THEIR EXTRACTED JUICES

BY

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AND

ASSISTANT SURGEON W J WOODHOUSE, I M D

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[Received for publication, July 10, 1933]

THIS investigation is the continuation of the work published by Wats (1929), Wats and White (1931), and Wats and Eyles (1932)

It was ascertained by Wats and Eyles (1932) that the sprouted *mung* (*Phaseolus mung*) was best amongst the various kinds of seeds tested—as to its tenderness when consumed raw ('salad'), its high antiscorbutic value, and the ease with which it germinated. These experiments were carried out in Upper Burma with the pulse available locally but it was found that at least three distinct kinds were available in the Calcutta bazaar. It is well known that the vitamin values of the various food-stuffs differ according to the variety of the articles, and the environment in which they have been grown (McCarrison, 1930, Bracewell, Hoyle and Zilva, 1930). It was to determine whether the above statement was applicable to the other varieties of this 'dal' that the work was undertaken. Guinea-pigs, as usual, were used for assaying the antiscorbutic values.

The varieties of mung—There are three kinds of this seed available in the Calcutta bazaar differing in size of the seed according to the source from which they are derived —

(a) The Chinese variety, imported from Singapore, has the biggest seed, requiring on an average five seeds to an inch, if these are laid lengthwise. The

bulk of the seed is about double the size of the Bengal, and about one and a half times that of the Upper India variety. The average length of the sprouted radicle is 3.6 cm.

(b) The Upper India variety is grown in the Punjab, Rajputana, etc. The seed holds an intermediate position between the China and Bengal types, about six seeds when laid lengthwise covering an inch. In bulk the seed is one and a half times that of the Bengal kind. The average length of the sprouted radicle is 3.3 cm.

(c) The Bengal variety is a local product of the province, on an average eight seeds when laid lengthwise cover an inch. The seed is much smaller than either of the above. The length of the sprouted radicle is 2.75 cm.

Method of sprouting—For comparative values Wats and Eyles (1932) suggested a standard period of 48 hours for soaking and sprouting of the seeds. These experiments were carried out in Maymyo during the cold weather and no deterioration of the sprouts was noticeable, but in Calcutta towards the end of March it was observed that the radicles of the seeds became speckled with brown coloration and withered at the tips. It will be seen from the graphs that all the experimental animals fed on these began to lose weight (on or about 30th day of the experiment). The substitution of 5 hours soaking and 19 hours germination gave rise to normal sprouting with consequent rise in the weight of these animals. It is noteworthy that covered receptacles, due to the moist atmosphere, favoured sprouting. The length of the sprouts was longest when samples were kept in semi-darkness, but for our experiment the process was carried out in an ordinary laboratory room. It is not possible to lay down any standard period, the time of the soaking and sprouting depending on the climate. The ideal should be to obtain healthy, creamy white radicles without any brown speckling or withering of the tips. It may be added that the Upper India variety contained about 20 per cent unsproutable seeds, which were similar in looks to the good ones. This is, probably, due to adulteration by unscrupulous dealers, hence a careful selection was necessary to employ only sprouted seeds for the experiments.

Juices of the sprouted mung—Although germinated mung has shown the highest antiscorbutic value amongst the various seeds tested so far, the objection has been raised to its use that the consumption of the raw material is liable to set up gastric irritation. One of us has frequently consumed about two ounces of the fresh sprouted dal as salad and repeated the experiment on other persons of various ages without any digestive disturbances occurring. During the cooking process, apart from the destruction of the vitamin C, the product is rendered very tough and even prolonged boiling does not soften it. To obviate this difficulty and at the same time to eliminate indigestible material such as cellulose, etc., it was decided to extract the juice from the germinated samples and test its value. The latter was passed through a mincing machine and the juice extracted by passing through fine muslin, 5 per cent of citric acid with an adequate amount of sugar was added and oleum lemonis was used for flavouring the mixture. This artificial lemon juice was quite pleasant to the palate but owing to its acidity the experimental animals refused to take it, even when mixed with large quantities of basal diet. The antiscorbutic

value of the preparation in this form could not be determined for this reason. Hence for the experiment citric acid and the lemon flavour was omitted and the pure juice was obtained by pressing through muslin and allowed to stand in a glass cylinder for an hour, the supernatant watery juice being subsequently pipetted off and used for experiment. If immediate feeding was not possible, liquid paraffin was added to form a layer on the top.

EXPERIMENTS

Basal diet—Guinea-pigs were given a basal diet consisting of bran and oats (equal parts) with 30 per cent of crushed gram (*Cicer arietinum*) as described by Wats and White (*loc cit*). To meet any criticism as to deficiency of vitamins A and D, the basal diet in certain animals was supplemented by one drop of Haliverol (vitamins A and D concentrate supplied gratis by Messrs Parke, Davis & Co., to whom our thanks are due).

Experimental groups

GROUP 1 *Control animals*—

- (a) Two guinea-pigs on basal diet as above
- (b) One guinea-pig on basal diet with one drop of Haliverol

GROUP 2 *Groups given germinated mungs*—

- (a) *China mung*—One guinea-pig on basal diet supplemented by a drop of Haliverol with 3 grammes of the germinated dal
- (b) *Upper India mung*—One guinea-pig on 3 grammes of sprouted seed and basal diet with one drop of Haliverol
- (c) *Bengal mung*—Two guinea-pigs (i) 3 and (ii) 4 grammes of the sprouted seeds with basal diet containing one drop of Haliverol

GROUP 3 *Groups given extracted juice from China and Bengal variety of mung*—

- (a) *China mung*—Three guinea-pigs on basal diet with Haliverol plus 3, 4 and 5 c.c. of the extracted juice
- (b) *Bengal mung*—Five guinea-pigs on basal diet without Haliverol plus 1, 2, 3, 4 and 6 c.c. of the extracted juice

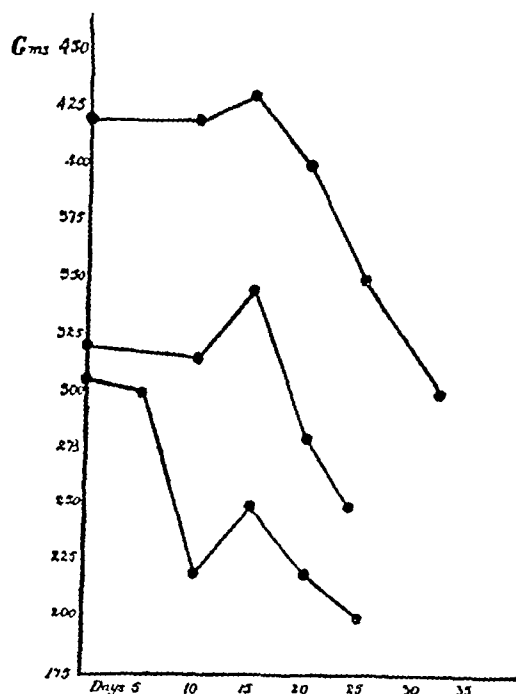
RESULTS

GROUP 1

The control animals without Haliverol died on 24th and 32nd day having lost 65 and 120 grammes from their initial weight of 320 and 420 grammes respectively.

The control animal with Haliverol died on the 25th day having lost 110 grammes from the initial weight of 305 grammes. The usual signs of scurvy were noted.

GRAPH 1
Controls (basal diet)



GROUP 2.

- (a) *China mung*—The animal on 3 grammes maintained its initial weight until the 37th day when it began to lose weight. On substitution on the 42nd day of 24 hours sprouted dal in place of dal sprouted for 48 hours the rise in weight was noticed at the next weighing, i.e., 49th day. The experiment was stopped on the 70th day when the animal had gained 50 grammes over its initial weight (300 grammes).
- (b) *Upper India mung*—The animals rose steadily in weight till the 30th day and went on losing weight until the 42nd day. On substitution of the germinated dal as above [see (a)] the weight rose from the 49th day onwards. The experiment was stopped on the 70th day, the animal's weight showing an increase of sixty grammes over its initial weight (300 grammes).

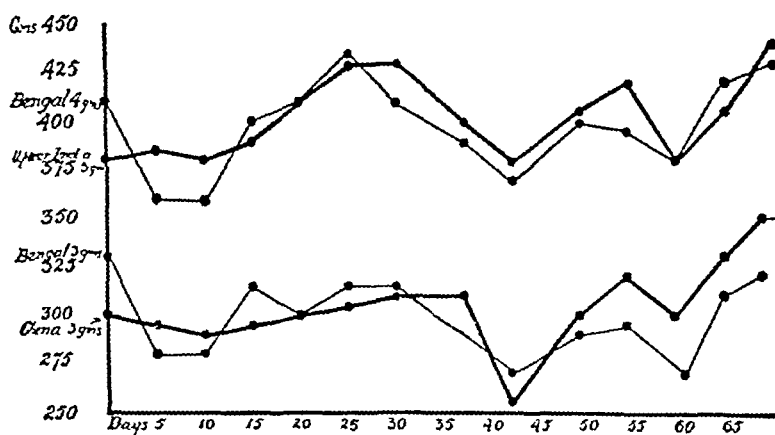
(c) *Bengal mung* —

(i) After an initial drop for ten days the animal more or less maintained its weight until the 30th day. The same phenomenon as in (a) and (b) was noticed regarding the loss and gain of weight. The experiment was stopped on the 70th day, the animal having lost ten grammes from its initial weight (300 grammes) but showing no signs of scurvy.

(ii) The animal on 4 grammes after the initial drop in weight for ten days rose steadily till the 25th day when a similar phenomenon as detailed above was noted. The animal at the termination of the experiment (70th day) had gained 15 grammes over its initial weight of 410 grammes.

GRAPH 2

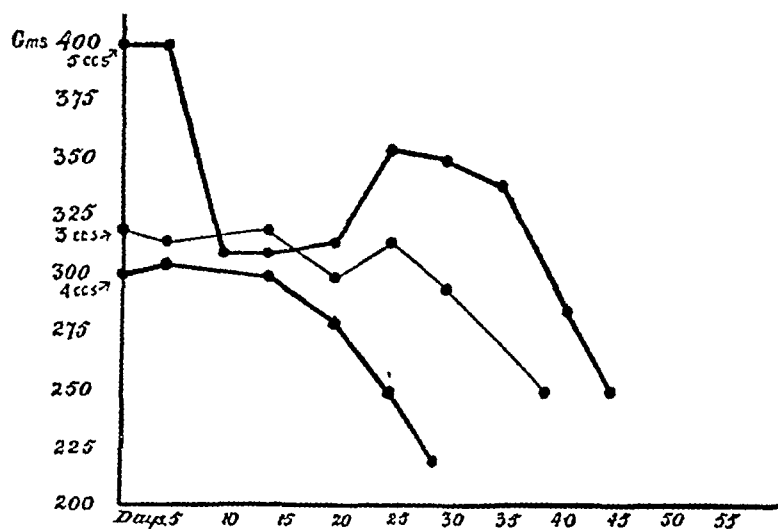
Sprouted *mung* dals (basal diet and Haliverol)

GROUP 3 *Extracted juice* —

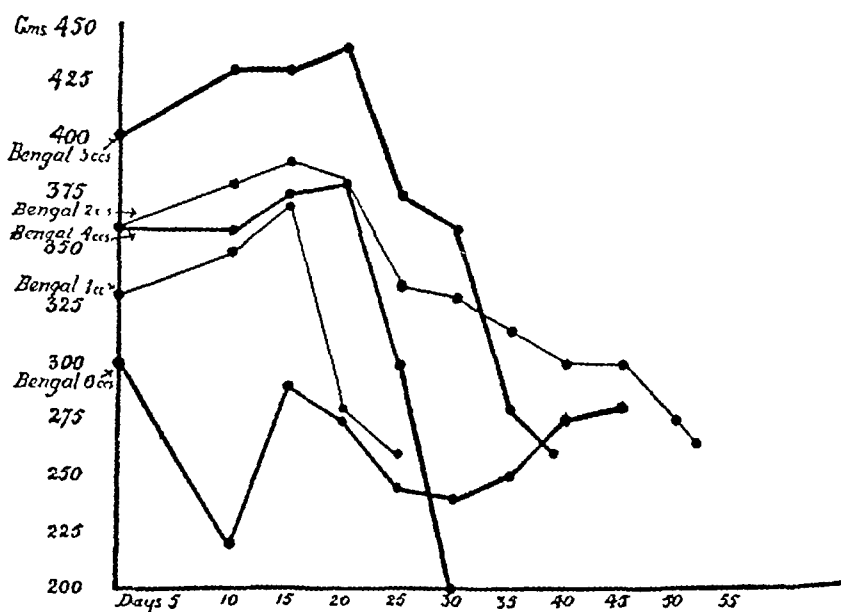
(a) *China mung* — All the three animals after varying ups and downs in weight died on 37th, 26th and 42nd day respectively.

(b) *Bengal mung* — The above also is applicable to all the animals except the one on 6 c.c., the death taking place on the 25th, 52nd, 40th and 30th days respectively. The animal on 6 c.c. was showing clinical symptoms of scurvy, hence the experiment was stopped.

GRAPH 3

China mung (expressed juice, basal diet and Haliverol).

GRAPH 4

Bengal mung (expressed juice and basal diet).

The protective value of the sprouted dal is well shown but the failure of the extracted juices to protect guinea-pigs from scurvy was puzzling, especially when the sprouted seeds, weight for weight, had been found to be as potent as orange or lemon juice. The following experiments show that the destruction of the vitamin C is due

to the action of the potent oxidizing enzymes present in the juice. After mincing some of the sprouted seeds the juice was extracted by passing the mass through muslin. After filtering, a few c.c. of each were added to eight test-tubes, four of which were boiled for a few minutes.

(a) To two of the tubes (1 boiled and 1 unboiled) a few drops of a 1 per cent solution of benzidine in 50 per cent alcohol were added. On standing a slight blue colour was noticeable in the unboiled juice only.

(b) The above experiment was repeated with two more samples (one being boiled) by substituting a solution of p-phenylenediamine hydrochloride in water instead of benzidine. A greenish-blue colour developed in the unboiled specimen.

(c) The experiment was similar to (a) except that a few drops of hydrogen peroxide were added. An immediate blue colour was noted in the specimen.

(d) Similar to (b) but few drops of hydrogen peroxide were added. An immediate greenish-blue colour was noted.

From the above experiments the presence of oxidase and peroxidase was inferred.

SUMMARY AND CONCLUSIONS

The basal diet with oats, bran and crushed gram worked quite satisfactorily, the animal thriving well without any intestinal disturbances. The addition of concentrates of vitamins A and D to the basal diet in some experiments did not seem to make any appreciable difference in the maintenance of weight of the animals or defer the onset of the special symptoms of scurvy.

Three grammes of the sprouted grain of each of the varieties of *mung* tested were found to be an adequate antiscorbutic for guinea-pigs.

The failure of the juices obtained by crushing the germinated seeds to protect against scurvy is attributable to the presence of oxidase and peroxidase enzymes.

Further experiments were carried out and it was ascertained that even 10 c.c. of the freshly extracted juice of the Upper India variety of *mung* acted as a poor antiscorbutic, symptoms of scurvy supervened on the 50th day of the experiment.

ACKNOWLEDGMENTS

We acknowledge our thanks to Lieut.-Colonel A. D. Stewart, I.M.S., who gave us all facilities to carry out the work at the All-India Institute of Hygiene and Public Health, Calcutta.

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COMPARATIVE BIOCHEMICAL FINDINGS IN BLOOD OF NORMAL AND MALARIA-INFECTED MONKEYS

BY

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[Received for publication, September 30, 1933]

BIOCHEMICAL aspects in the malaria-infected monkeys were studied in the hope that some significant information might become available to add to the knowledge of the ætiology and pathology of the disease. The first difficulty encountered was the absence of any data recorded to show the limits of normality of the chemical constituents of the blood in monkeys.

The species of animal infected with a parasite encountered in a Singapore monkey was *Macaca mulatta*, samples of blood were collected after a night's fast. In the following investigation the results obtained from normal monkeys, together with those infected with malaria, are recorded side by side for comparative purposes. In several cases the normal and the post-infection results are from the same animals as shown by the serial numbers. Some observations of other workers in human malaria have been referred to under the appropriate headings, these may prove of interest as the normal biochemical figures for the blood of human beings and monkeys are, with minor exceptions, similar.

Blood-sugars—Sinton and Hughes (1924) in the course of a study of the functional capacity of the liver in human malaria found the fasting blood-sugar in fifteen cases to vary from 146 mg to 75 mg per cent—the average value being 105 mg per cent. Ruge *et al* (1929) investigating all varieties of malaria found little evidence of deviation from the normal in both pyrexial and apyrexial cases and in no case was the value greater than 130 mg per cent.

476 *Findings in Blood of Normal and Malaria-Infected Monkeys.*

Technique—The colorimetric method of Folin and Wu was employed

Results of present investigation

The blood-sugar values found in normal and infected monkeys are summarized below —

TABLE I

Blood-sugar-values given as mg per 100 c c of blood

NORMAL		INFECTED WITH MALARIA		
Serial number	Sugar	Serial number	Intensity of infection	Sugar
29	87.5	30	Parasites +, blood taken a day after 2 grains of quinine intramuscularly	81.6
30	100	38 I	30,800 parasites per c mm, chiefly schizonts	97.5
30	109	38 II	1,200,000 parasites per c mm, chiefly schizonts, blood taken a day after atebirin 0.1 g	96.0
41	96	38 III	No parasites	114
45	111	41	Parasites + + +, blood taken a day after 2 grains of quinine intramuscularly	95
Average	100.7	Average		96.8

The values found are in agreement with those recorded by various observers in human malaria and warrant the conclusion that no noteworthy alteration in the level of the blood-sugar occurs in the malaria-infected monkeys

Non-protein nitrogen—Yamanaka (1927) states that urea and residual nitrogen are generally increased in the blood in human malaria. Several observers have noted an increase of urea as a result of accelerated catabolism, depending on the severity of the infection.

Technique—Colorimetric method of Folin and Wu was employed

TABLE II

Non-protein nitrogen-values given as mg per 100 c c of blood

NORMAL		INFECTED WITH MALARIA		
Serial number	Non protein nitrogen	Serial number	Intensity of infection	Non protein nitrogen
29	33.8	41	Parasites + + +, blood taken a day after 2 grains of quinine intramuscularly, red blood corpuscles $3\frac{1}{2}$ millions	30
30	38.0	47	2,016,000 parasites per c mm, chiefly schizonts	38.8
30	25.0	49 I	42,340 parasites and $5\frac{1}{2}$ millions red blood corpuscles per c mm	35.9
41	29.7	49 II	No parasites, atebryn 0.1 g given daily three days before collection of blood, red blood corpuscles 5 millions per c mm	40.0
45	27.3	53	207,200 parasites, chiefly mature schizonts, red blood corpuscles 5 millions per c mm	30.0
Average	30.76	Average		34.9

It would be noted that in the normal monkeys the maximum and minimum values lie between 25 and 38 mg respectively and the average value comes to 30.8 mg per cent. There is little evidence to show any abnormality in this respect in the infected monkeys.

Phosphates—Estimations of the inorganic content were made on the plasma by the method developed by Benedict (1924)

TABLE III

Inorganic phosphates—values given as mg. per 100 cc of plasma

NORMAL			INFECTED WITH MALARIA BUT NOT TREATED			INFECTED WITH MALARIA AND TREATED WITH ANTI MALARIAL REMEDIES.		
Serial number	Inorganic phosphates	Serial number	Intensity of infection	Inorganic phosphates	Serial number	Intensity of infection	Treatment	Inorganic phosphates
29	4.3	38 I	30,800 parasites per c mm, chiefly schizonts	5.1	30	Parasites +	2 grains of quinine intramuscularly	6.2
30	4.4	47 I	2,016,000 parasites per c mm (rings, schizonts and a few gametes)	3.6	38 II	1,200,000 parasites per c mm, chiefly schizonts	Atebrin 0.1 g intramuscularly	4.9
30	3.6	49 I	42,340 parasites per c mm, chiefly mature schizonts, 5½ millions red blood corpuscles per c mm	4.9	38 III	No parasites, normoblasts and basophils present in blood	Blood taken three days after atebrin treatment	3.5
41	3.96	52	412,800 parasites per c mm, all rings	3.5	41	Parasites	Blood taken after 2 grains of quinine given intramuscularly on the previous day	6.8
45	2.63	53	207,200 parasites per c mm, chiefly schizonts	2.15	45	882,000 (chiefly schizonts) per c mm	Neo quinine (a synthetic remedy for trial) given on three successive days before collection of blood	4.7
					47 II	Nil, red blood corpuscles 3½ millions, anisocytes, normoblasts, basophils and demilune cells present	2 grains of quinine intramuscularly on three successive days previously	6.2
					48	921,600 parasites per c mm, red blood corpuscles 3½ millions, basophils and anisocytes present	2 grains of quinine intramuscularly a day previously	6.6
					49	No parasites, red blood corpuscles 5 millions	Atebrin 0.1 g intramuscularly on the two previous days	4.5
					50	No parasites, red blood corpuscles 3½ millions, scanty normoblasts, achromia and basophilus present	Do	5.0
Average	3.78	Average		3.85	Average			5.27

Amongst the normal animals the maximum and minimum values of inorganic phosphates lie between 2.6 mg and 4.4 mg with an average of 3.7 mg per cent. The infected animals treated with anti-malarial remedies show a distinct increase above the normal average without showing a corresponding increase in the non-protein nitrogen. The animals infected with malaria but not treated in any way show the inorganic phosphates at almost the same level as the normal ones, hence the increase in the treated animals should be ascribed to the quinine, atabrin, etc., and not to the malarial infection. Whether the mobilization of inorganic phosphates has anything to do with the metabolism of these remedies or is merely the result of tissue destruction caused by the intramuscular injection, would be an interesting study.

Calcium—Whitmore (1928) reports serum calcium values of 13.5 per cent and 16.4 per cent in two cases of human malaria in blackwater fever subjects. Control cases, however, likewise gave higher values than the accepted normal (9 mg to 11 mg per 100 cc of serum).

Technique employed in present investigation was based on the work of Kramer and Tidsall, Tidsall, and Clark and Collip. The results are as follows—

TABLE IV

Calcium—values given as mg per 100 cc of serum

NORMAL		INFECTED WITH MALARIA		
Serial number	Calcium	Serial number	Intensity of infection and treatment if any	Calcium
31	12.7	41	Parasites + + +, 2 grains of quinine intra muscularly given a day previous	11.4
30	10.4	45	882,000 (chiefly schizonts) per c mm	10.5
41	11.0	47	2,016,000 parasites per c mm (rings, schizonts and a few gametes)	12.1
Average	11.36	Average		11.33

As no marked difference was evident in the three cases recorded above the investigation was not pursued further.

Cholesterol—In human cases de Paulo Santos (1916), using a modification of Gregaut's method, found a diminished cholesterol content in malaria. Porak (1918) found hypocholesteræmia as a characteristic of malarial blood and the decrease in

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amount proportionate to the gravity of the case Crespin and Zaky (1919), in an analysis of 30 cases, found that the blood cholesterol fell below normal prior to the malarial paroxysm Borel *et al* (1926), also using the method of Gregaut, describe hypocholesterinaemia in all varieties of malaria

Technique employed was that described by Myers and Wardell (1918), oxalated blood plasma being used for estimation

The results are as summarized below —

TABLE V
Cholesterol—values given as mg per 100 c c of plasma

NORMAL		INFECTED WITH MALARIA		
Serial number	Cholesterol	Serial number	Intensity of infection	Cholesterol.
29	119	30	For intensity of and treatment please see Table III (inorganic phosphates)	88.2
30	121	38 I		70.0
30	133	38 II		75.0
41	114	38 III		72.0
45	120	41		75.0
		45		88.8
		47		100.0
		48		85.7
		50		80.0
Average	121.4	Average		81.63

There is a marked decrease in the cholesterol content of the animals infected with malaria, it is difficult to say definitely but the most likely explanation is that this hypocholesteræmia reflects the degree of anæmia caused by the malarial infection

SUMMARY.

1 In monkey malaria no noteworthy changes take place in the blood-sugar, non-protein nitrogen or calcium

2 There is a decrease of cholesterol content in plasma.

3 No marked increase of inorganic phosphates takes place in the untreated infected cases but a distinct rise is evident in the infected animals treated with atabrin, quinine, etc Its significance is not clear.

We would like to record our thanks to Lieut-Colonel R N Chopra, I M S, Officiating Director, School of Tropical Medicine, Calcutta, who gave us every facility to carry out the work. We are also grateful to Mr A C Roy, M Sc, and other colleagues of the Biochemical Laboratory of the School for valuable suggestions.

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ON* ELECTROCARDIOGRAMS

Part I.

BLOOD-PRESSURE AND ELECTROCARDIOGRAPHIC CHANGES WITH MUSCLE EXTRACT

BY

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THE action of drugs on living cells possesses certain unique features. Many drugs act at extremely low concentrations, and the specificity of action observed is without parallel in any other science. The effect of a drug ultimately reduces itself into its activity on living cells. The cell is a highly complex system and possesses certain properties, one of which is its electrical charge. Moreover, when cells are excited, there is a variation in this charge, and on the variation of electrical charge of tissue-cells of the heart-muscles during its contraction is based electrocardiography. Though electrocardiography has already proved to be of immense value in diagnosis of different cardiac conditions, it is generally known that a knowledge of the relation between the physiological condition of the tissue-cells of the cardiac muscle and some aspects of the records on an electrocardiogram is still indefinite. For example, to take an extreme case, we see that Lewis (1925) ascribes the final or T deflection in the electrocardiogram to electric forces generated by the return of the ventricular muscle from its active to its resting stage, while Pardee (1925) considers the deformed T wave, found in the electrocardiogram in coronary thrombosis and myocardial infarction, to be produced by the 'reaction of repair occurring around the anæmic area'. Wilson, Macleod and Barker (1931) write that, in cases of myocardial infarction, a current of injury is present during diastole and that the final ventricular deflection is not written entirely by the currents produced by recovery but in part by the current employed to neutralize the current of injury which disappears partly or completely when the injured muscle becomes active. Thus, it is clear that the voltage or height of a particular wave in an electrocardiogram is one of the less-known variables, the magnitude of which depends in an

unknown way on many physico-chemical and the corresponding physiological factors

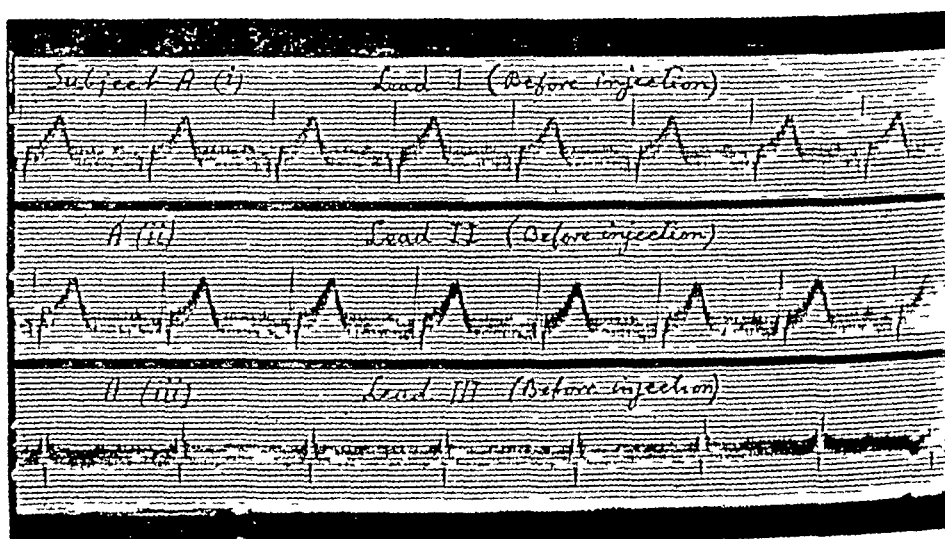
It is well known that administration of muscle extract to human subjects decreases the blood-pressure and we have also seen (*see below*) that it decreases the height of the waves in an electrocardiogram. The purpose of the present paper is mainly to elucidate some of these obscure points so that we may find out a relation between the variables mentioned above

EXPERIMENTAL DETAILS

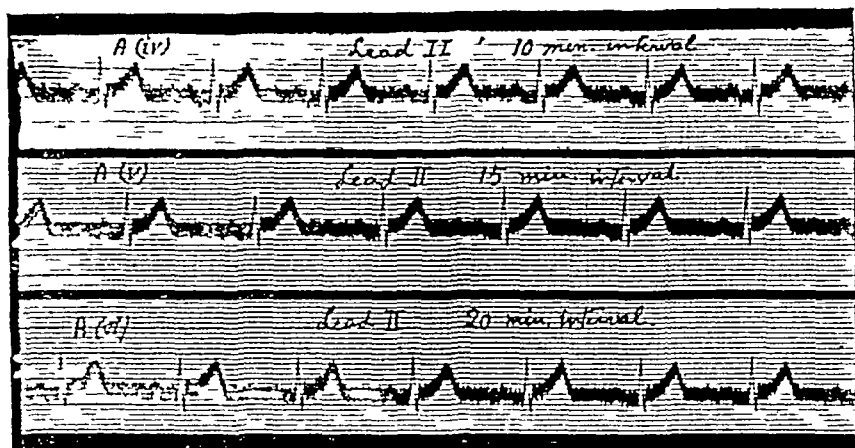
The muscle extract was prepared following the method of Drury and Szent Gyorgi (1929-30), with slight modifications

The muscles used were the skeletal muscles of a he-goat. The extract obtained was concentrated by evaporation on a water-bath kept at 45°C to 50°C . One c c of the extract represented 10 grammes of the skeletal muscle and 0.5 c c of this was injected intramuscularly into different normal human subjects. By preliminary experiments it was determined that the effect of the extract on different individuals manifests itself about 5 to 10 minutes after injection and disappears completely after 20 to 30 minutes. So at intervals of 10, 15 and 20 minutes after injection, the blood-pressure was measured and electrocardiograms were taken. As it was clear that there was no fundamental difference in the changes produced in the first, second and third lead after administration of the muscle extract, we only recorded the second lead, though all the leads were taken before injection to ensure that the subject's heart was healthy. Two of the typical electrocardiograms (lead II) are given for illustration and the results on blood-pressure as well as those on the voltage of P, R, T waves in electrocardiograms of different individuals are tabulated below. Here t denotes the time interval in minutes after injection and the properties at $t = 0$ represents the values before any muscle extract was administered

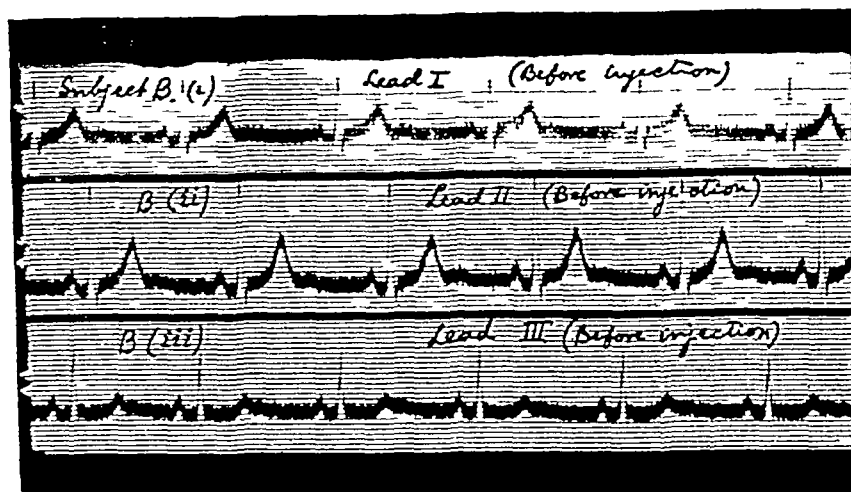
ELECTROCARDIOGRAM I



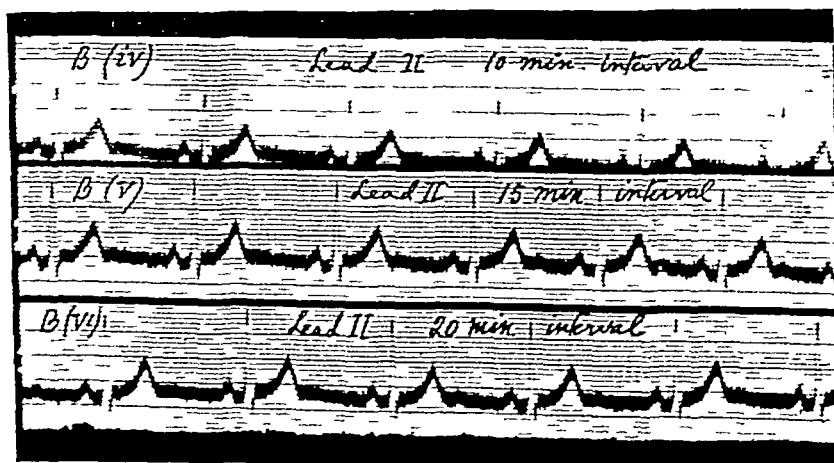
ELECTROCARDIOGRAM II



ELECTROCARDIOGRAM III



ELECTROCARDIOGRAM IV



TABLE

Showing the variation of blood-pressure and voltage with time

Patient and age	B P				P in millivolts				R in millivolts				T in millivolts			
	at <i>t</i> (min)				at <i>t</i> (min)				at <i>t</i> (min)				at <i>t</i> (min)			
	0	10	15	20	0	10	15	20	0	10	15	20	0	10	15	20
H M 38	110/80	102/76	102/76	110/80	0.45	0.35	0.4	0.45	1.2	0.9	1.0	1.0	1.05	0.75	0.9	0.95
A-I M 26	106/76	98/70	96/70	90/70	0.7	0.6	0.5	0.6	2.4	2.0	2.0	1.8	0.93	0.8	0.8	0.7
M M 28	104/76	98/62	100/72	102/72	0.65				2.3			2.0	1.0			0.9
H M 28	110/85	98/68	98/78	104/80	0.5	0.45	0.42	0.4	1.1	1.05	1.2	1.3	0.55	0.55	0.55	0.6
A-I M 21	110/80	98/70	98/70	102/74	0.6	0.4	0.55	0.60	2.3	1.5	1.95	2.1	1.15	0.7	0.95	1.0
H M 31	106/78	102/82	104/84	104/78	0.6	0.5	0.5	0.6	2.3	1.8	2.3	2.3	0.75	0.65	0.7	0.75
M M 40	94/68	90/64	94/68	94/68	0.5	0.45	0.5	0.5	1.6	1.4	1.6	1.7	0.7	0.6	0.5	0.7
M M 28	100/80	96/80	94/80	98/78	0.6	0.62	0.6	0.7	1.8	1.95	2.1	2.1	0.75	0.72	0.78	0.9
M M 45	95/68	88/62	88/62	88/62	0.6	0.5	0.5	0.55	1.8	1.52	2.05	1.9	0.7	0.6	0.7	0.8

No change in P-R interval Pulse rate slow—most markedly at the 15 minute interval

DISCUSSION

(A) *General results*

From an inspection of the results on arterial pressures and electrocardiograms, it will be observed that in almost all cases, except one or two, the voltage of P, R, T waves becomes less, simultaneously with a diminution of blood-pressure after the intramuscular injections of muscle extract, and these effects are transient, lasting only for 20 to 25 minutes at best. The pulse-rate is also diminished and it is most marked at the 15-minute interval. This bradycardia is due to a prolonged T-P interval. There is, however, no change in the P-R interval—which is rather surprising in view of the results of Drury and Szent Gyorgyi (*loc cit*) who have found a prolonged P-R interval in guinea-pigs after intravenous injections of extracts from 0.5 g of muscle. This discrepancy in results might be due (1) to different methods of administering the extract, (2) to a difference in the subjects chosen, or (3) to a different source for obtaining the extracts. The slowing of the pulse-rate is not observed in patients put on muscle extract treatment in the hospital. We are therefore of opinion that with therapeutic doses of muscle extract there is no organic change in the heart.

(B) *Cause of the depression of wave-amplitudes after injections of muscle extract*

There is, however, a distinct though short-lived variation in the voltage of the waves after injections of muscle extract. It is difficult to suggest the significance of these changes in physiological terms. Of all the variables in an electrocardiogram, this factor, i.e., the low or high voltage of wave-amplitude, is least understood, we might go further and say that its relation to a particular physiological condition of the heart is not clear, though some authors [see, for instance, Speckmann and Rich (1931), *cf* also Turner (1932)] have tried to associate this condition in human subjects (under no drug) with myocardial degeneration. After the administration of a particular drug, if a condition is set up which disappears after a short time, it must be admitted that the change produced is not organic. It is certain that the amplitude of the waves (i.e., the voltage) apart from any physiological meaning, signifies only a potential set up within the heart during the process of contraction. It is just possible that a drug can either increase or decrease the potential, so registered, by increasing or decreasing the surface density of electrical charge on the cardiac muscle, if we accept the theory of limited potential as advanced by Lewis (1922) to explain the relation between the contraction of the heart and the corresponding electrocardiogram. It appears that some of the constituents of muscle extract might decrease the negativity of the potential, set up during contraction, being adsorbed by the tissue-cells. It is evident that this adsorption is reversible and therefore when fresh venous blood comes into the heart the adsorbed ions are gradually replaced till the tissue-cells regain their normal state. This view is further supported by the fact that when initially the surface density of the charge (i.e., the amplitude or voltage of the

waves) is high, there ought to be a greater diminution of charge (i.e., the amplitude or the voltage of the waves) at a constant time with the same concentration of the muscle extract, due to increased attraction for and consequent greater adsorption of the active ions. That this is so will be evident from the time-voltage and blood-pressure time curves shown in Figures 1 and 2 (cf Patients Nos 5 and 7 in the Table)

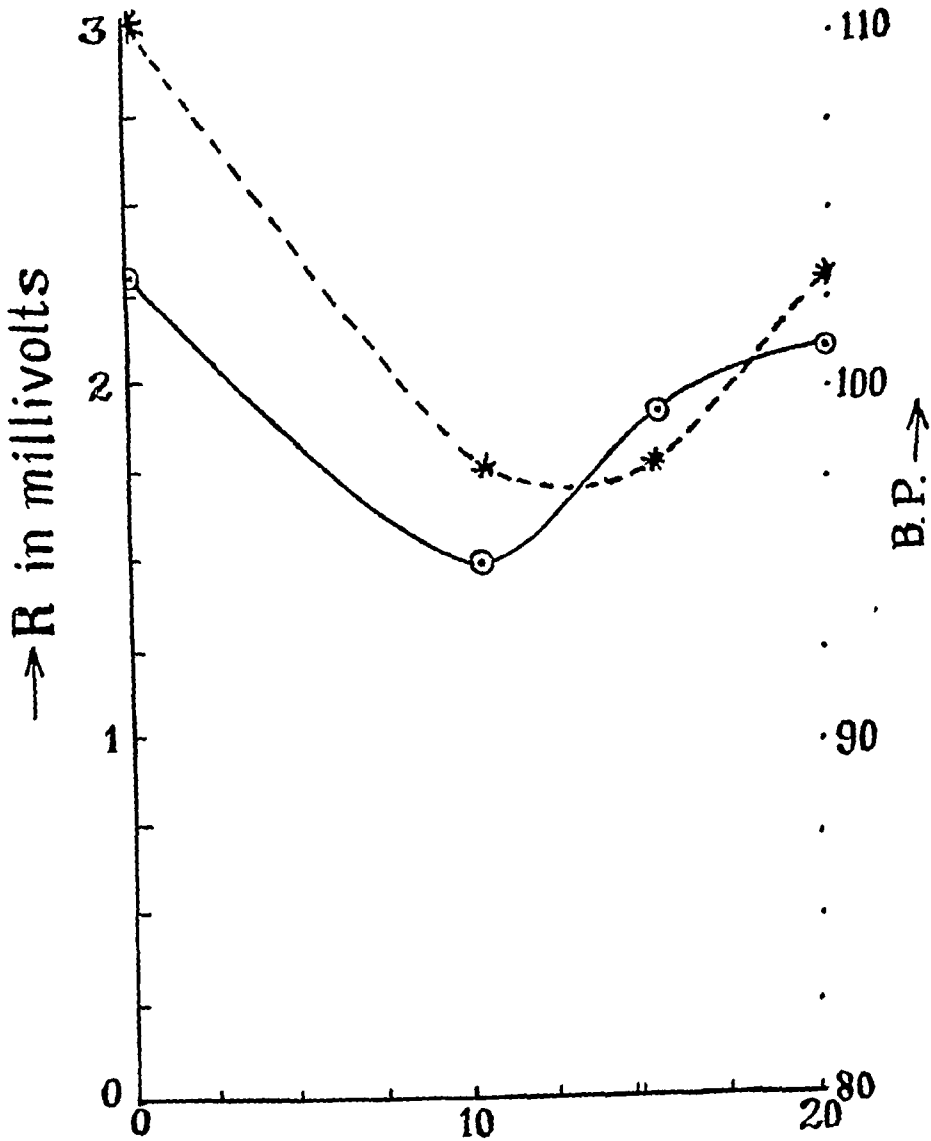
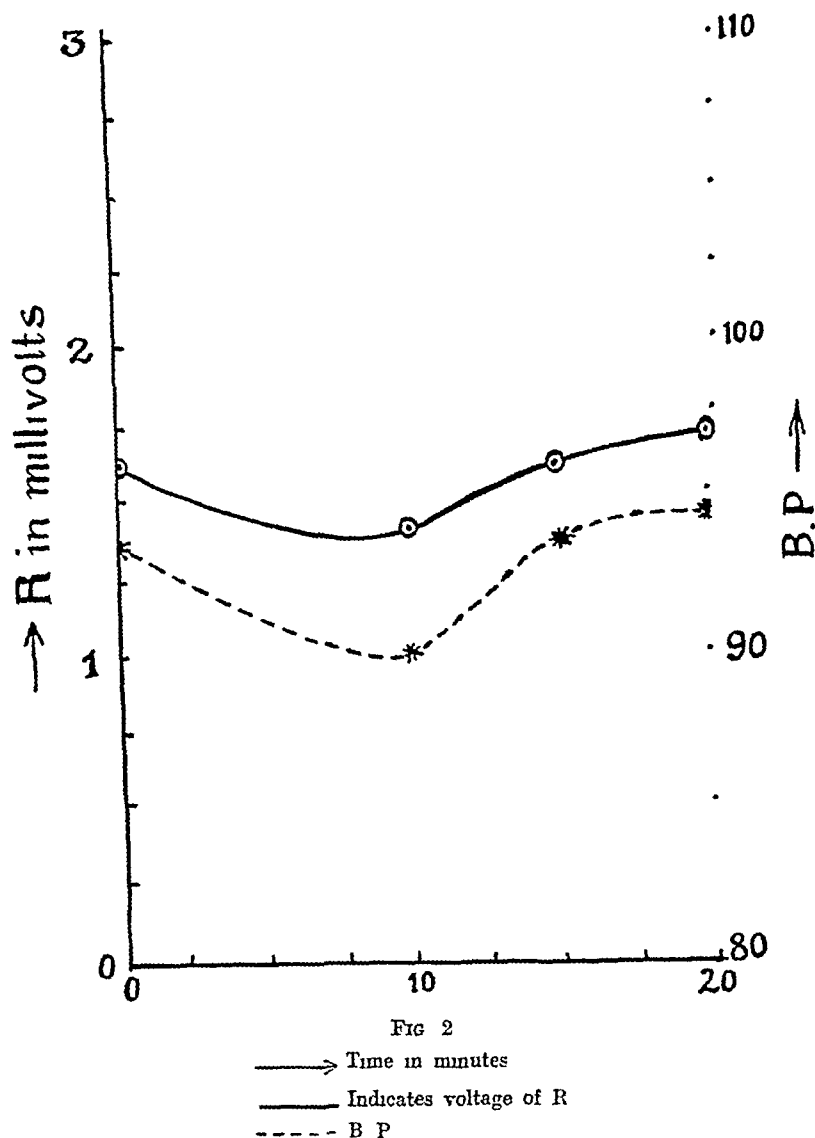


FIG 1

—→ Time in minutes
 — Indicates voltage of R.
 - - - - B P



(C) *Relation between diminution of the wave-amplitude and decrease in blood-pressure after injections of muscle extract—a possible physico-chemical explanation of the physiological phenomena*

A fall in blood-pressure after injection of muscle extract may be due to —

(1) Stimulation of the vagal mechanism, (2) depression of the sympathetic nerve-endings in the heart or the peripheral vessels, (3) direct depression of

the cardiac muscle, (4) peripheral vaso-dilatation, by direct action on the musculature

That it is not due to (1) has been shown by the fact that the fall in blood-pressure with muscle extract is obtained even after atropinization. It has also been seen that with a concentrated dose (0.1 c.c. for a cat of 2.5 kilo) the auricles and the ventricles both dilate though there is no change in the force of the heart. There is also dilatation of the peripheral blood vessels and slowing of the pulse-rate. Hence the fall in the blood-pressure must be attributed to a direct action on the cardiac muscle and the peripheral blood vessels. Thus, it is clear that, if we desire to find out a relation between the amplitudes of P, R and T waves (especially of R) in an electrocardiogram and the observed fall in blood-pressure on a rational basis, we have first to explain the dilatation of the blood vessels from the physico-chemical point of view and connect the change in the particular set of physico-chemical variables (responsible for vaso-dilatation) with the variation of the charge of the tissue-cells of the cardiac muscle (responsible for diminished amplitude of the waves).

It has already been stated that the immediate effect of administration of muscle extract is cardiac- and vaso-dilatation and a consequent diminution in arterial pressure. It is therefore apparent that the change in any physico-chemical property, if such occurs must be immediate and must therefore take place on the interfaces of the blood stream and the endocardium of the heart as well as the intima of the arteries. Such a property is the interfacial tension and it is conceivable that, in concentrated doses, the release of the tension is sudden and pronounced so that there is immediate cardiac- and vaso-dilatation. This change in interfacial forces in addition to other factors is intimately bound up with a change in the electric potential of the interface. Thus, for instance, Macallum (1912) says that the nerve impulse is always accompanied by a change in electric potential which is initiated by progressive distribution of potassium ions along the course of the axon and this change in potential would occur as often as a change of tension could take place on the surface of the nerve-cells. On the basis of this hypothesis, anaesthesia and narcotism are to be interpreted as involving a reduction in surface tension of all the cells affected but especially of nerve-cells.

Further, it is known that the change in potential at the interface (Freundlich—potential or electrokinetic potential) is dependent on the desorption or adsorption of ions from or on the surface and it is also known that dilatation of the arteries and the heart is dependent on the elasticity of the cardiac muscle and the arterial walls. It is therefore necessary to understand the effect of a variation of charge of the tissue-cells (on the endocardium and the intima) on the elasticity of the respective tissue-cells. If the charge is only affected on the surface, then the effect of elasticity (longitudinal) will be partly neutralized, if the charge of the cells is increased and somewhat augmented when it is diminished, provided, of course, there is no change in either the permeability of or the osmotic forces inside the cell-membrane. Now, if it be true that tissue-cells can be stretched, and will then regain their original form, one does not know how much of this effect is due to surface tension, and how much to the elastic properties of the cell-membrane and how much if any to the elastic properties of the interior protoplasm (Heilbrunn, 1928, *cf* Seifriz, 1924, 1926). Evidently then the elasticity of the tissue-cells or arterial walls or muscles, as we know by its various manifestations in the human body, is *not* the

same as we signify it in pure physics. Thus, it is clear that through a change in the potential at the interface, the elasticity (lateral at the arterial walls and the cardiac muscles), comprising surface forces at the interface of cells and tissue-fluids, cohesive forces between the individual cells, and elastic properties of the cell-membrane and of interior of the protoplasm, will be altered. Here in this particular case of muscle extract, the elasticity (as defined above) is diminished as a net effect of the above-mentioned variables, possibly and mainly through a reduction in potential at the interface and in charge of the adjoining tissue-cells and thus there is a diminution in arterial blood-pressure.

A very significant similarity is also observed between the rate of diminution of the potential or electrical charge of a colloid in presence of electrolytes and the rate of decrease of blood-pressure with a particular drug (which is known to depress the arterial blood-pressure). When the initial density of charge of a colloid is high, the diminution in charge is greater for a particular concentration of any electrolyte than when the initial density of charge of the colloid is low, if the density of charge of the colloid is sufficiently low to start with, it cannot be depressed further even with oppositely charged polyvalent ions (*cf* Mukherjee *et al*, 1928). The similarity is obvious, for with a particular concentration of any drug (which depresses blood-pressure), the fall in blood-pressure is greater when the initial blood-pressure of the subject is high compared to that when it is low, and even with a powerfully active drug, the blood-pressure cannot be lowered, if it is initially very low. It is therefore obvious that the fall in blood-pressure is also related to the electric potential at the interface of the blood stream and the endocardium and the intima.

Thus, both the diminished blood-pressure and the decreased amplitude of the waves are due to a diminution of the electric potential and interfacial tension at the cell-liquid interface and hence a change in records on an electrocardiogram does not always mean a change in inherent properties, both physiological and physico-chemical, of the constituent tissue-cells of the cardiac muscle as a whole, but may sometimes only indicate alterations of some of these variables at the interface of the blood and the cardiac muscles.

SUMMARY AND CONCLUSIONS

(1) It has been shown that, after about 10 minutes of administration of muscle extract into normal human subjects, there is a diminution of blood-pressure and also slowing of the pulse-rate (10 to 20 minutes more).

(2) Electrocardiograms, taken under these conditions, show that there is a decrease in the amplitude of the waves P, R, T and that the slowing of the heart-rate is due to a prolonged T-P interval.

(3) Decrease of amplitudes has been attributed to a decrease of the electrical potential of the cardiac muscle during its contraction by the adsorption of some of the constituent ions of muscle extract, diminution of blood-pressure has been attributed to a decrease in electric potential and interfacial tension at the interface intima-blood causing thereby dilatation of the peripheral blood vessels.

(4) A change in records on an electrocardiogram does not always mean a change in inherent properties of the constituent tissue-cells of the cardiac muscle as a whole,

but may sometimes only indicate alterations of some of these variables at the interface of the blood and the cardiac muscles

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ACTION OF THE INDIAN DABOIA (*VIPERA RUSSELLII*) VENOM ON THE CIRCULATORY SYSTEM

BY

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THE Indian daboiia or *Vipera russellii* is ranked with the Indian cobra as one of the most dreaded snakes of the Orient. Ewing reported that as many as 20,000 deaths occur every year in India from snake-bite and that the majority of these were produced by the bite of cobra and daboiia. The study of the pharmacological action of both these venoms was therefore taken up some time ago and the results obtained with the venom of the Indian cobra *Naja naja vel tripudians* have already been published (Chopra and Chowhan, 1931).

A perusal of the literature shows that there has been considerable doubt regarding the action of the viper venom. Wall and Cunningham first studied the physiological action of the venoms of both cobra and that of Russell's viper and later Calmette and Noguchi worked out the hæmolytic and coagulating properties of these venoms. Lamb and Hanna (1903) reported that death in case of viper bite is produced by extensive intravascular clotting, while in chronic cases there is a marked decrease in the coagulability of the blood and extensive hæmorrhages in the serous cavities. Rogers working on the physiological action of and the antidotes to colubrine and viperine venoms, reported that death is due to intravascular clotting in the finer blood vessels and when the poisoning is slow it is due to circulatory failure and emboli in the lungs.

Of the Indian vipers the important species is *Daboia russellii* (*Daboia elegans*). It is also called 'Uloo bora' (cham viper) in and around Calcutta (Fayrer) and is the same as 'Tic polonga' of Ceylon. The Indian daboiia is from 3 to 5 feet in length and so far as colours and patterns go it is strikingly beautiful. The body is light-brown in colour and of a rich hue. Extending on either side and throughout the entire length there are three rows of large brown rings, bordered with white, yellowish or black colour, enclosing dark brown or even red spots. In some species the rings of the back unite and form a continuous chain. Like the rest of viperine snakes it has a movable jaw, canaliculated fangs, triangular head and a short tail. On account of its beautiful and gaudy coat this snake has been named *Daboia elegans* or *Echedna elegans*.

Russell's viper is very common in the thickly populated parts of Bengal and Assam. It is found in Rajputana and practically throughout the plains of India. In Burma, Ceylon, Siam, Sumatra and Java it is frequently seen. It has also been observed in the valleys of Kulu and Kashmir up to an altitude of 5,000 to 6,000 feet above sea level, though it is rarely seen elsewhere above an altitude of 2,000 to 4,000 feet. It is generally of quiet and peaceful habits and prowls about at night in search of prey which consists generally of mice, rats, frogs, lizards, ants, eggs, etc. It attacks man in self defence and only when provoked. When ready to attack it produces a loud hissing sound which can be heard from a distance of 20 to 25 feet.

Venom of Indian daboa—Mitchell and Reichert showed that the intense local irritant effect produced by the bite of the viper is due to large quantities of globulin (as much as 25 per cent) present in the venom, the albumoses, which produce mainly nervous symptoms, are present in very small quantities. The viper bite always leads to local irritation, intense pain, with abscess formation and gangrene at the site of injection of the venom. The systemic effects are hæmorrhagic effusions in the splanchnics and ascending paralysis in the central nervous system. Acton and Knowles (1914) showed that daboa venom contains a hæmorrhagin which destroys the endothelial lining of the finer blood vessels and consequently gives rise to ecchymosis and extravasation of blood, a cytolyisin which dissolves the white and red blood corpuscles, a fibrin ferment causing intravascular and extravascular clotting leading to pulmonary embolism and death from asphyxia. The venom loses its toxicity to one third if it is mixed and incubated with formalin. Lamb and Hanna (*loc cit*) showed that by heating a 0.1 per cent solution of daboa venom, the protein substances are coagulated and the property of intravascular clotting of the venom is completely destroyed. The blood remains fluid in cases of death caused by daboa venom, probably on account of the proteolytic action of the venom on the blood fibrin. Alcohol precipitates the neurotoxic and coagulant substances.

EXPERIMENTAL

Toxicity and minimum lethal dose—It has been noted that the action of daboa venom is not so rapid and potent as that of the cobra venom. The daboa venom has more or less a localized action giving rise to severe irritation and abscess formation at the site of inoculation. This is attributed to the localized clotting of blood and fixing of the venom at the site of the bite, thus producing a severe local action. The toxicity of the daboa venom was worked out on the following animals—

1 Wild rats and house rats (*Mus musculus*) A one per cent solution of the venom was injected intravenously into the dorsal vein of tail of the common house rat. On an average 3 mg to 5 mg of the venom killed the animals weighing 350 g within 2 to 4 hours. Before death the rats become restless, and dyspnoeic, breathing becomes more and more rapid and the animals become very weak and unable to move. The symptoms are those of profound shock and respiratory failure.

2 Guinea pigs Eight to ten mg of the daboa venom given subcutaneously in animals weighing 500 g to 600 g produce death in 24 hours. The symptoms produced are practically the same as in rats.

3 Rabbits Two to three mg given subcutaneously killed the animal within 24 hours. Large doses such as 5 mg to 10 mg produce death in a few hours, the main symptoms being those of shock and failure of respiration. When a sublethal dose is given, hæmatoma, subcutaneous hæmorrhages and even abscess formation results at the site of injection. In a few cases hæmorrhages occurred in the vagina.

4 Frogs are able to tolerate as much as 20 mg per 30 g body-weight. This remarkable tolerance may possibly be due to the pulmocutaneous type of respiration which enables the amphibia to excrete the venom quickly and completely.

The lethal dose of the venom when injected subcutaneously is considerably larger than when it is given intravenously. This is due to the fact that the venom forms local thrombi in the small blood vessels and therefore its absorption is delayed. Moreover, the action of the venom is localized and the general effects are less marked. The reason why the lethal doses were mostly worked out by the subcutaneous and intramuscular routes was that, in nature, the venom is injected by these routes.

The fatal dose for man has been calculated to be approximately 42 mg within 24 hours. It has been found that 7.5 mg of the venom were fatal in monkeys weighing 25 kilos. Lamb estimated that the maximum dose a full grown Russell's viper can inject is about 150 mg to 250 mg of the venom at a single bite while the average dose given at a single bite is about 72 mg.

Post mortem examination—Necropsies were done in all the animals in which the venom was injected. The right side of the heart was full of almost black fluid blood and both the auricles and the ventricle on the left side were empty and in a state of systole. The lungs were collapsed and cyanosed.

and showed petechial hemorrhages and infarctions. The blood vessels and the viscera were engorged with venous blood and the peritoneal cavity was full of sero sanguinous fluid. The liver and the spleen were generally congested, and the kidney showed a marked hyperemia of the cortical blood vessels, there may be extravasation of blood in the glomeruli. We did not find any signs of gangrene in any part of the body. The animals which had received sub lethal doses were restless and dyspnoeic at first and later became lethargic. This was followed by paralysis of the limbs and acute inflammation at the site of injection.

PHARMACOLOGICAL ACTION

Cats weighing between 2 and 3 kilos were mostly used for experimental work, frogs, rats and rabbits were also used where necessary. The venom of the Indian daboia used in this work was obtained through the courtesy of the Director of Haffkine Institute, Bombay. It was kept in hermetically-sealed ampoules in the dark. For experimental purposes a 1 in 1,000 solution was prepared by dissolving the venom in physiological saline solution. Constant and vigorous shaking for some time is necessary to dissolve the scales of the venom and a frothy and slightly opalescent solution is finally obtained. Injections were given intravenously into the femoral vein of the anesthetized animals.

Local action—Locally the application of 1 to 2 per cent solution does not produce any marked effect on the intact skin, the mucous membrane, e.g., of conjunctiva, is irritated and becomes red. When injected deeply into the muscles inflammation and even abscess formation may result in 24 to 48 hours.

Action on the digestive system—Our experimental work on cobra venom has shown that it is of the nature of an enzyme and the daboia slightly accelerates salivary digestion of starch in concentrations of 1 in 10,000 to 1 in 20,000 in man. During the act of deglutition in snakes the secretion from the glands is automatically squeezed out and the venom is swallowed along with the food. If in a snake the venom is removed by milking and the reptile is artificially fed protein digestion is very greatly interfered with.

It is said that a weak degree of immunity can be acquired against the venom by feeding animals with gradually increasing doses of this substance. This might mean that the venom is absorbed from the alimentary canal. It has also been stated on the other hand that the venom is destroyed in the large intestines in very much the same way as strophanthum. Our experiments show that the venom of Russell's viper is not absorbed from the gastro-intestinal tract when administered by the mouth. A number of cats were given 8 to 10 times the lethal dose of venom orally without any ill-effects whatever.

Intravenous injections of the venom produce a marked increase in the intestinal volume (Graph, fig 6). On the intestines *in situ* and on the isolated pieces of the intestine perfused in Dale's uterine bath, daboia venom in concentrations of 1 in 300,000 to 1 in 5,000,000 slightly increased the tone and the peristaltic movements.

In India snake venom is habitually taken by some people with the idea of increasing their resistance against poisons. It is also combined with opium and other narcotic drugs under the impression that it potentiates their effects, but there is no evidence to support these views.

Action on the respiratory system—Records of intratracheal respiration and lung volume. Small doses of the venom (0.1 mg per kilo) produce a slight increase in the

frequency and amplitude of the respiratory movements. With larger doses (0.2 mg to 0.3 mg per kilo) the respiratory movements become irregular and spasmodic, sometimes followed by complete cessation and convulsions. A small dose of adrenalin at this stage raises the blood-pressure to its normal level and the respiratory distress is relieved immediately.

The effect on the respiratory centre was investigated by isolating a strip of the diaphragm and connecting it to a writing lever through a system of pulleys according to the technique described by Thomas and Frank. The blood supply of this strip is almost entirely cut off and any impulses that come from the respiratory centre must come through the phrenic nerves only. Intravenous injections of the venom in such animals produced a slight increase in the tone, and stimulation of the automatic movements followed by twitchings and relaxation of the strip (Graph, fig. 4). This indicates that there is a slight initial stimulation of the respiratory centre, probably due to anæmia of the medulla as a result of fall of blood-pressure. The centre then tries to send extraordinary transitory impulses to all the accessory muscles to revive the respiratory mechanism. The fact that administration of adrenalin improves the respiration shows that the venom does not have any direct action on the respiratory centre and in this way it differs from cobra venom. Chopra and Ishwariah (1931) have given ample experimental proof that the main action of cobra venom in lethal and sub-lethal doses in animals is on the respiratory centre, the effect being one of an initial stimulation followed by paralysis. Death with cobra venom occurs from respiratory failure, while with viper venom the main cause is circulatory failure.

The pulmonary pressure is definitely increased after an intravenous injection of the venom in doses of 0.1 mg to 0.2 mg. Another interesting fact observed is that rapid coagulation of blood in the cannula in the pulmonary artery, which is often an annoying factor in experiments, was never met with after injection of the venom, thus showing that the coagulability is reduced by these injections.

Uterus—Intravenous injections of 0.1 mg to 0.3 mg have little effect on the movements of the virgin uterus of the cat *in situ* or isolated uterus in a Dale's bath.

Action on the circulatory system—As death after dabara-bite is mainly to be ascribed to circulatory failure, the action of the venom on this system was studied in some detail.

Blood-pressure.—The systemic blood-pressure is dependent upon the total quantity of blood circulating in the body, the work done by the heart and the condition of the peripheral blood vessels. The vascular areas of the viscera, skin, muscles and brain play an important part in regulating the blood-pressure and equilibrium is maintained by contraction and relaxation of the arteries, arterioles, capillaries and veins. If this balance is in any way disturbed the blood accumulates in the heart or in the blood vessels and capillaries. The blood-pressure recorded by a manometer is merely the sum total of the physiological balance of all the above factors and varies with their antagonistic or additive action.

In our experiments it was noted that a small dose of the venom (0.5 mg to 0.1 mg), injected intravenously into a cat weighing 2 kilos, produced a slight initial rise of blood-pressure followed by a gradual fall amounting from 20 mm to 30 mm of mercury. The curve however gradually returned to normal in about 30 to 40 seconds. With larger doses (0.2 mg to 0.5 mg) the fall was more pronounced and

comparatively rapid, the blood-pressure remaining permanently at a lower level than the normal (Graph, figs 2, 5 and 6). Rapid administration of large doses produced a sudden fall of blood-pressure and the animal may die suddenly of convulsions and heart-failure. If the dose of the venom is gradually increased, it is observed that the animals develop a sort of tolerance to it. When once the blood-pressure has reached its lowest level after a dose of the venom, further administration of much larger doses does not produce any effect on the blood-pressure. In case of animals having an initially low blood-pressure, the response to the venom is not so marked and the fall is comparatively small (Graph, figs 1, 3 and 4).

Similar results were obtained in animals whose brain and spinal cord were destroyed showing that the cerebral and medullary centres play a minor part in the production of the fall. Paralysis of the sympathetic and vagal nerve-endings with ergotoxin and atropine does not alter the fall in blood-pressure after administration of the venom. When the blood-pressure is low, large doses of normal saline infused intravenously or a small dose of adrenalin raise the blood-pressure to its normal level.

We are able to confirm the observations made by Ishwariah and David (1932) that no fall in blood-pressure is produced after histamine. After 2 mg to 3 mg of histamine when the blood-pressure has reached its lowest level daboia venom does not lower the blood-pressure at all below the level at which it stands. It can also be shown that after large doses of daboia venom histamine does not produce its usual effect on the blood-pressure.

It would appear from this that the venom has a local and paralysing action on the vessels closely resembling that of histamine. Once the capillaries are paralysed to their fullest extent they fail to respond to these substances any more. Injections of adrenalin or transfusion of large quantities of normal saline, which increase the volume of the circulating blood and thus fill the heart better, would raise the blood-pressure and thus give a better chance of recovery from the effects of the venom.

The heart—In the cat, the volume of the heart as measured by a cardiometer, shows a slight and transient increase (dilatation). The myocardiographic tracings (Graph, fig 3) show that the heart becomes slow, and the amplitude of the beats of both the auricles and the ventricles is decreased. If large doses are given (0.2 mg to 0.5 mg) the heart becomes irregular and finally the auricles and ventricles stop in diastole. Sometimes the heart becomes irregular and continues to beat intermittently for a long time.

On the frog's heart, when the brain and spinal cord have been destroyed, the application of the venom in concentrations of 1 in 10,000 produces little or no effect. On the isolated heart of the cat, perfused with Ringer-Locke solution at pH 7.2, the venom in concentrations of 1 in 1,000,000 and 1 in 500,000 produced practically no effect, concentrations of 1 in 100,000 to 1 in 50,000 slightly depressed and reduced the amplitude of the beats. Still higher concentrations such as 1 in 50,000 to 1 in 10,000 made the isolated heart of both rabbits and cats irregular and intermittent, concentrations such as 1 in 2,000 to 1 in 1,000 produce a rapid depression of the heart which finally comes to a standstill.

It would appear from these experiments that daboia venom has no marked direct effect either on the myocardium or on the nervous apparatus of the heart.

Whatever effect is produced is due to the changes brought about on the vascular system

Volumes of visceral organs—The cause of fall of blood-pressure and the situation where the blood accumulates when the blood-pressure falls, were next investigated. The volume of the intestines is markedly increased when the blood-pressure falls (Graph, fig 6). The limb volume shows no marked changes while the decrease in the kidney and spleen volume runs parallel with the fall in the blood-pressure to begin with (Graph, figs 2 and 5).

The fall of blood-pressure and its maintenance at a lower level after a minimum lethal dose of dabara venom has been administered, according to some workers, is due to enfeeblement of the intestinal musculature. Extensive hæmorrhages occur due to injury of the endothelial cells of the blood vessels and due to non-coagulability of the blood, both of which lead to exudation of blood into the tissues. Acton and Knowles (1914) showed that death in case of viper venom was due to cardiac failure and later from multiple hæmorrhages. The post-mortem examination of animals who were given fatal doses of the venom in our experiments showed extensive engorgement of the abdominal viscera and the presence of sero-sanguinous fluid in the peritoneal cavity was a constant factor. That the blood accumulates in the abdominal viscera has been shown by the fact that in animals which have been completely eviscerated injections of the venom produced little or no fall of blood-pressure.

The part played by the blood vessels in the fall of blood-pressure was further investigated.

Action on the blood vessels—With a view to studying the direct effect of the venom on the involuntary muscle of the blood vessels, frogs were perfused according to Trendelenburg's technique.

A frog was pithed and fixed on the frog board and after opening the abdomen and chest a very fine cannula was passed into the arch of the aorta before its branching. This cannula was connected by a rubber tubing to a Mariotte bottle filled with frog's saline solution (0.7 per cent) and the rate of the flow was regulated by a screw clamp fixed on to the tubing. The Mariotte bottle was closed with a rubber stopper containing a long glass tube going to the bottom of the fluid so that the air bubbles entering the fluid kept it properly oxygenated and maintained a constant level of the pressure. The number of bubbles entering the bottle in a fixed time gave an estimate of the discharge of the fluid. The inferior vena cava was cut across to form an outlet for the venous blood. The legs of the frog were crossed and were so arranged that when the frog was hung vertically, all the perfused fluid trickled at one point only. The number of bubbles per 30 seconds recorded when different doses of the venom were perfused though the general circulation showed a definite constriction of the blood vessels.

In warm blooded animals similar experiments were performed by perfusing the blood vessel of a limb or the mesenteric vessels. Cats were anaesthetized with chloralose and fine cannulae were fixed into the femoral vein and artery of one of the limbs. The cannula in the artery was connected with the Mariotte's bottle containing warm Ringer's fluid with a little defibrinated blood. The number of drops emerging from the femoral vein were counted per 30 seconds. Previous to perfusion of the limb, care was taken to exclude every possible means of collateral circulation by ligaturing inside the abdomen the common iliac artery going to that limb. In the case of the mesenteric blood vessels, one of the main branches of the superior mesenteric artery and its corresponding vein were secured and a fine glass cannula passed into each of them. The collateral circulation to this mesenteric arch was carefully occluded by tying all the vessels communicating with this arch. The mesenteric blood vessels were perfused with Ringer Locke solution which was oxygenated and kept at body temperature. The inflow of the bubbles and the outlet of drops of fluid were counted per 30 seconds.

A perusal of Tables I and II will show that the vessels of the limb are definitely constricted, while those of the splanchnic area are slightly dilated, when perfused with different concentrations of the venom.

Fig 1

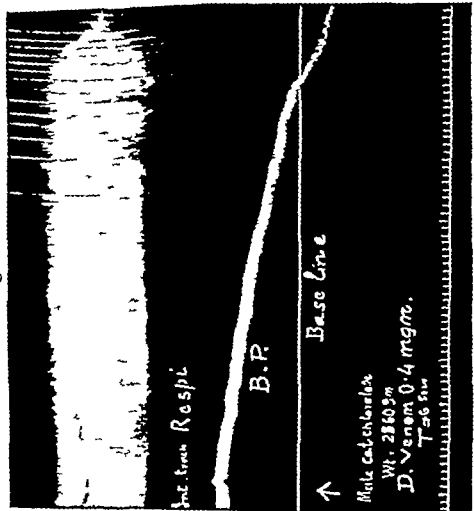


Fig 2



Fig 3

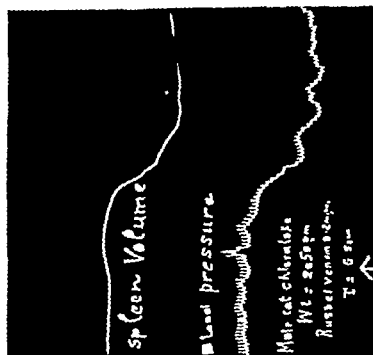
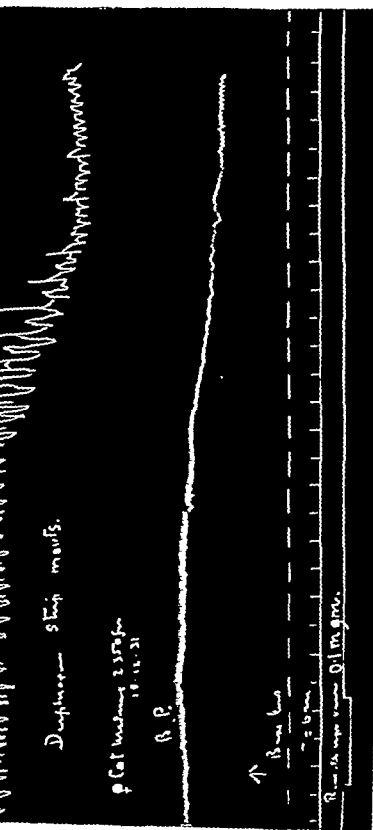
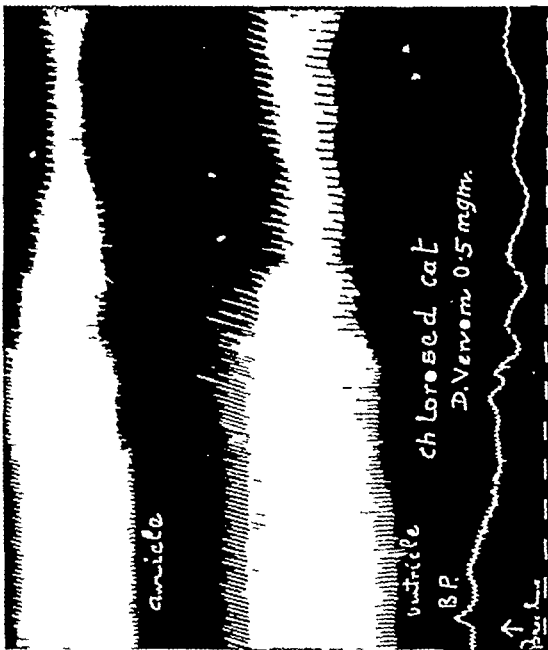


Fig 4

Fig 1 Male cat, 2,860 g, chloralose. Time 6 seconds. Upper intratracheal respiration and lower, carotid blood pressure 0.4 mg of daboia venom given intravenously. Note a marked irregularity and spasms followed by complete cessation of the respiratory movements. The pressure 0.02 mg of daboia venom given intravenously. Fig 2 Female cat 1,080 g, chloralose. Upper kidney volume and lower carotid blood pressure. Fig 3 Cat chloralose, myoelectric tracings and blood pressure. 0.5 mg of daboia venom given intravenously. Note the reduction of the amplitude of the auricular and ventricular beats and fall of blood pressure. The beats become irregular and slow, but there is a tendency to recovery. Fig 4 Female cat, 2,350 g, chloralose. Chest open and artificial respiration. Record of movements of strip of diaphragm according to Frank's technique. 0.1 mg of daboia venom given intravenously. Note an initial slight increase of tone and spasmodic contractions followed by relaxation. Fig 5 Male cat, 2,050 g, chloralose. Upper, carotid blood pressure and lower, carotid blood pressure. 0.2 mg of daboia venom given intravenously. Effects same as in Fig 2. Fig 6 Male cat, 1,890 g, chloralose. Upper, intestinal volume

Fig 5

Fig 6

TABLE I

Perfusion of limb vessels of the cat

Number	DOSE OF DABOIA VENOM IN MG									REMARKS
	Animal	Saline control	0.02	0.03	0.04	0.05	0.07	0.15	0.25	
1	Cat No I	16 drops	14.0 drops	0.03	0.04	0.05	0.07	0.15	0.25	Average number of drops in 30 seconds Do
2	Cat No II	19.6 ,	19.6 "		9.0 drops		8.8 drops			
3	Cat No III	10.3 "		9.0 drops	17.2 "		16.0 "		9.8 drops	

TABLE II

Perfusion of mesenteric vessels of the cat

Number	DOSE OF DABOIA VENOM IN MG						REMARKS
	Animal	Saline control	0.03	0.06	0.15	0.25	
1	Cat No I	32	29	27	26	19 seconds	Average time taken for 10 bubbles
2	Cat No II	15	19	41		bubbles	Average number of bubbles in 30 seconds

Perfusion of arterioles—The action of the venom on arterioles was also studied

The superior mesenteric artery was perfused in the usual manner, but the attachments of the mesentery with the intestines were severed by cutting all along the intestinal and mesenteric junction. In this way the solution flowed through the mesenteric artery, its branches and arterioles and oozed out before it passed into the capillaries and veins. The effect of the drug on the arteries and its fine branches, excluding the capillaries, was thus obtained. The venom was injected into the rubber tubes leading from the Mariotte bottle to the artery and the readings were always taken about a minute after the administration of the drug so as to give sufficient time for the venom to act.

There is a distinct reduction of the fluid showing that the arterioles are contracted under the effect of daboia venom.

Action on the capillaries—The toxic action of the venom on the vessels is not only confined to the arterioles, but also extends to the capillaries though in an opposite way. Such also is the case with poisonous bases and toxins produced by bacteria, etc. The capillaries of the splanchnic area appear to show a predilection to these effects and hyperæmia and escape of blood from the gut vessels by leakage from the capillary wall is very marked.

It has been claimed by some that the branched cells, known as Rouget's cells, placed immediately outside the capillary wall have the power of altering the lumen of the capillaries. This has been denied by others and contractile power of the endothelial cells themselves is said to be responsible for their dilatation and contraction. It is quite possible that the endothelial cells stretch during acute dilatation of the capillaries and leakage of the plasma may occur in this way. In whatever way this may occur there is no doubt that the cause of shock is dilatation of capillaries by which 'the animal may be said to bleed into its own dilated capillaries'. An examination of the frog's web shows how large is the number of capillaries there as compared with the arterioles and how much more blood is contained in them. Lister, Langley and Krogh showed that under ordinary conditions the whole of the capillaries of a particular region are not filled with blood. Some of these are empty and apparently in a contracted condition. More capillaries come into evidence and become filled with blood if there is comparatively higher blood-pressure in the arterioles as, for example, after exercise. Dale and Richardson have experimentally demonstrated that histamine produces a profound fall of blood-pressure by dilatation of the capillaries of the splanchnic area and not of the arteries or arterioles. In the case of daboia venom we have been able to demonstrate that the fall is due to dilatation of the capillaries although the arterioles are actually contracted.

The relation between arterioles and capillaries is twofold. Firstly, though some of the capillaries supplied by an arteriole may become constricted, others may not. Secondly, the arteriole may be constricted, while the capillaries may be widely dilated, and vice versa. The study of the action of a drug on the size of capillaries by means of the perfusion method is very difficult. The ordinary technique of perfusion of the vessels gives a record of the condition of the arteries, veins and arterioles only, but not of the capillaries. The perfusion of the systemic vessels and arterioles with daboia venom, however, shows that the vessels are either slightly constricted or are not affected at all. It has also been observed that after large doses of histamine daboia venom has no effect on the blood pressure and vice versa. There would thus appear to be a great similarity in the action of these two substances. The action of the venom on the capillaries, however, is difficult to demonstrate by means of perfusion experiments. We therefore devised the direct method of measuring the size of the capillaries with the help of a micrometer scale in a microscope. Krogh showed that the under surface of a frog's tongue, bladder and omentum, the pancreas of the rabbit and the tail of the tadpole, etc., form good subjects for study of the capillary circulation. When properly prepared, they appear quite transparent under the microscope and the blood is clearly seen circulating through the fine vessels and capillaries. One can watch the capillaries disappearing after exposure for some time and reappearing after some period. Our studies in this laboratory were mostly based on observations on the under

surface of the frog's tongue and bladder and some experiments were also done on the capillaries of the rabbit's mesentery

Technique—In a shallow Petri dish the paraffin wax is plated, and a semi circular hole is cut through the wax in the centre of the dish. The mesentery of an anesthetized rabbit is gently stretched over this hole and fixed with pins. A pledget of cotton wool soaked in normal saline is put on the edge of the hole in contact with the under surface of the mesentery to keep it moist. This preparation is placed on the stage of a binocular microscope and the finer vessels of this membrane are focused upon it. One eye piece of the microscope is fitted with a micrometer which can measure up to 10μ . The vessels visible in the field are sketched on a paper and one of the vessels is focused under the micrometer so that any changes in its shape and size can be measured. At this stage if a light scratch is made with a fine needle or a feather one or more capillaries will reappear upon the surface and the circulation in them is re established. Any changes in their size can now be easily observed. The venom is applied locally to the mesentery and the changes in the vessels carefully observed.

The effects were compared with such drugs as caffeine 1 in 10,000, histamine 1 in 10,000, adrenalin 1 in 10,000, urethane 1 in 10,000, digitalis, etc. Daboia venom like histamine produces a distinct and well-marked dilatation of the vessels while digitalis and adrenalin constricted them.

Capillary permeability—It has been shown that the capillary walls are very thin and consist of only a single layer of endothelial cells well adapted to allow a ready diffusion of substances through it. Water and non-colloid constituents pass through readily while colloids and proteins go through slowly. Krogh devised a method of studying the fractional osmotic pressure of the blood which enables an estimation to be made of the relative proportions of colloid particles of large and small size. The smaller the size of protein particles the greater is their diffusibility. Another important factor in this connection is that the permeability is increased under circumstances where an acute dilatation of the capillaries occurs and this may result in oedema of the tissues. Dale and Laidlaw (1919) showed that when a dose of histamine was given to a cat there was a rapid concentration of the blood and this was ascribed to the increased rate of the capillary leakage. The number of red blood cells and haemoglobin percentage is increased. In case of rabbits and cats treated with viper venom it was observed that there was a distinct increase in the red blood cells and a slight decrease in the white blood corpuscles. These effects were particularly marked in cats. The polycythæmia produced is not due to excessive red blood cell formation but due to excessive leakage of the blood plasma and a consequent concentration of the blood. When the capillaries are widely dilated and are in a highly permeable condition the outward passage of the fluid from the capillaries and rapid increase in the number of red blood cells can be watched under the microscope. The red blood corpuscles are seen to collect in masses and block up the capillaries causing stasis in the flow. The reverse occurs when water is being retained in the capillaries. These effects can be demonstrated on the surface of the frog's tongue after application of viper venom.

Lewis (1927) showed that pituitrin increases the tone of the capillaries and restores the blood-pressure in a cat which has been brought to a condition of profound vascular shock by administration of histamine. Dale produced evidence in favour of the view that adrenalin is normally responsible for this upkeep of the capillary tone. Histamine and adrenalin are physiologically antagonistic. Administration of adrenalin has been shown to overcome such shock by holding this oligæmia in abeyance. Adrenalin keeps up the tone of the capillaries and prevents capillary leakage. It keeps up the adequate amount of plasma in the blood in this way.

avoiding leakage and consequent shock. Both adrenalin and pituitrin produce similar reviving effects in experimental poisoning with daboia venom showing that capillary paralysis is responsible for excessive loss of fluid from the vascular system.

Action on the blood —Fontana thought that after viper-bite the blood becomes hæmolyzed and the heart and the large vessels are found full of dark fluid blood. Others consider that the blood coagulates at once when it comes in contact with the venom but is liquefied later on. Weir Mitchell considered that death occurs so rapidly after the bite that the blood has no time to become modified by the venom. It has been observed that the venom has hæmolytic constituents in the form of a fibrin ferment which is probably globulin in nature. This is responsible for the action produced on the red blood corpuscles, the leucocytes and the endothelium of the blood vessels. In our own experimental work on animals the blood remained fluid after death from daboia venom though the contrary has been reported by many observers.

Hæmolysis —Flexner and Noguchi believed that the erythrocytes are hæmolyzed by the venom only if they are not properly washed. The hæmolysis produced is due to the susceptibility of the red blood corpuscles which are insufficiently washed. Kyes (1910) showed that washing of the blood does not affect hæmolysis and that cobra venom does not dissolve the red cells of many species of animals. The property of producing hæmolysis is not possessed only by the cobra venom but is present in case of snake venoms generally. He tested 10 different species of venoms and showed that these have lytic action on the corpuscles of one or more species of animals and that the daboia venom hæmolyzed only human and guinea-pig blood. The same author further showed that the hæmolyzing property of the venom can be activated by the presence of lecithin or substances containing lecithin such as bile, hot milk, cephalin, etc. Ox's blood, for example, which was not hæmolyzed by daboia venom or cobra venom became hæmolyzed at once when lecithin (0.0035 mg per c.c.) was added. Cholesterol acts in an antagonistic manner to lecithin, and its presence in the blood prevents hæmolysis. It would appear that bloods which are hæmolyzed contain excessive quantities of lecithin in the plasma.

We worked chiefly with human blood. To 5 c.c. of normal saline 1 c.c. of blood was added, gently mixed and then centrifugalized at 2,000 revolutions per minute, when the blood corpuscles settled to the bottom of the tube. They were washed again with normal saline two or three times. A one per cent solution of the blood corpuscles was prepared and equal volumes of the suspension and of the venom solution mixed to make the final dilutions of 1 in 10,000, 1 in 20,000, etc. The mixture was kept at room temperature and the results read after 24 hours. Human blood was readily hæmolyzed in concentrations of 1 in 10,000 or more.

Coagulability of blood —It is still open to question whether the viper venom produces coagulation or hæmolysis of the blood. It has been suggested that the blood clots before death but the venom has a proteolytic action on fibrin and this produces a liquefaction of the blood again. It has also been said that proteolytic action manifests itself even in very small doses and that the compact clot formed soon becomes soft and is then re-dissolved completely. The daboia venom comes last in the range of coagulation.

We have tested the coagulation time of the blood in dogs and rabbits after administration of daboia venom. The capillary tube method was used. Fine glass capillary tubing 0.5 millimetre in diameter and about 10 cm in length was used. A rabbit's ear was shaved clean and one of the marginal veins was pricked, and when a big drop of the blood appeared one end of the capillary tube was dipped into it. The blood runs into the tube by capillary action. If the free end of the capillary tube is kept at a lower level almost the whole of the tube can be easily filled. The exact time when the blood is allowed to run into the capillary tube is observed by a stop-watch. After waiting for 1 to 2 minutes a centimetre of the capillary tube is snapped gently at the end distal to where the blood was allowed to run in. The tube is thus snapped at intervals of every 15 seconds and when a fine thread of fibrin clot is seen on breaking the tube, the time is recorded. This gives the normal coagulation time of the animal. Increasing sub-lethal doses of the venom were then injected subcutaneously and the coagulation time studied half an hour after each injection.

A reference to Table III shows that the coagulation time is distinctly increased after an injection of the daboia venom in sub-lethal doses. If, on the next day, a larger dose of the venom is given the coagulation time is still further increased. After the third dose the blood does not coagulate at all and remains fluid for a long time. A drop of this blood when examined under the microscope shows no changes in the corpuscles. Every time the rabbit's ear is pricked after administration of the venom an increased tendency to hæmorrhage is observed and it often becomes difficult to stop the oozing and a hæmatoma may form at the site of the prick. One rabbit died of hæmorrhage after administration of the venom for the bleeding could not be stopped after pricking the ear.

TABLE III
Coagulation time

Number	Animal	DABOIA VENOM INJECTED SUBCUTANEOUSLY				REMARKS
		Normal average	2 mg	2.25 mg	3 hours later	
		Mins Secs	Mins Secs	Mins Secs	Mins Secs	
1	Rabbit, 1,120 g	1 - 40	2 - 25	2 - 40	2 - 45	Coagulation delayed Do Do Do
2	" 1,525 g	2 - 20	2 - 40	2 - 51	3 - 5	
3	" 1,625 g	1 - 26		1 - 45	2 - 11	
4	" 1,670 g	1 - 21		1 - 54	2 - 8	
1	Cat No I	2 - 52		3 - 10	3 - 15	Coagulation delayed Do Do
2	" " II	2 - 41		3 - 5	3 - 10	
3	Dog " I	2 - 30	2 - 50	3 - 14	3 - 10	

Variation in the blood count after venom poisoning

Dalezenne noticed that all substances which reduce the coagulability of the blood dissolve the white blood corpuscles, setting free coagulant and anti coagulant substances, the former being retained by the liver while the latter remains in the blood and keeps it fluid. He further observed that venom in low concentrations disintegrates the white blood corpuscles but the red blood corpuscles are injured to a lesser extent. In higher concentrations the venom is destructive both to the red and white blood corpuscles. It should be remembered, however, that tremendous changes would occur in the counts of the peripheral blood owing to extensive leakage of the plasma and corpuscles from the capillaries of internal organs caused by the venom.

While the coagulation time was being tested in animals the total red and the white blood cell counts were also made. It was observed that there is no definite decrease in the white cells. The red cell count fluctuates, but there is an undoubted tendency towards increase of both the erythrocytes and leucocytes.

In previous papers (Chopra and Chowhan, 1931, 1932*a*, 1932*b*, Chopra and Ishwariah, 1931) while working on the action of venoms on unicellular organisms, the present authors showed that the mode of action in the case of the Indian cobra (*Naja naja*) and the Indian daboia (*V. russelli*) is entirely different. While the former acts on the rudimentary neuromotor apparatus in *Paramœcium caudatum*, the latter venom has an entirely selective action on the endothelial cells of the vascular system. Since the unicellular organisms have no formed vascular system the paramœcia escape the toxic effect of the viper venom while it is very vulnerable to the action of cobra poison. Our investigations recorded in this paper show that the viper venom acts on the endothelial layers of the blood vessels and particularly has a selective action on the walls of capillaries only.

SUMMARY AND CONCLUSIONS

In case of viper venom the hæmorrhagic phenomena appear at the outset of the poisoning and are very extensive in character. Death is preceded by spasmodic and irregular respiration, convulsions and asphyxia indicating the involvement of the vagal centre owing to deficient blood supply. In all post-mortem examinations recorded, the lungs show symptoms of asphyxia, petechial hæmorrhages and infarction. The right side of the heart is full of dark blood and the left side is empty and tonically contracted. In frogs the venom produces less harmful effects. In animals died of daboia poison the kidneys show inflammation, mottling of the cortex, hæmorrhages, etc. The serous cavities such as the pericardium, peritoneum, pleura, etc., are full of sanguinous fluid, probably produced by injury to the delicate endothelial cells of the capillaries leading to excessive leakage of the blood into the tissue spaces and the serous cavities.

Daboia venom has a marked tendency to produce thrombosis and gangrene at the site of the bite and death is due to secondary shock. The systemic blood vessels, especially the peripheral ones, are found to be contracted and those of the splanchnic area are widely dilated as in histamine shock. That the nervous centres are not much affected is shown by the fact that in decerebrated animals exactly the same results are produced. The fall of blood-pressure can be

overcome by warmth, pituitrin, adrenalin and large doses of saline, either by constricting the vessels or increasing the total quantity of the blood. This clearly shows that the fall of blood-pressure is neither central nor cardiac in origin. If, however, the action is prolonged these measures are of no avail since the normal relative permeability of the vessel walls to the protein constituents of the blood is lost. The capillary leakage goes on to such an extent that anything injected leaks out of the vessels.

The symptoms of shock in daboia poisoning are not due to reflex impulses but are due to the local dilatation of the capillaries of the splanchnic area. There is enormous engorgement of the abdominal viscera and that the collapse goes hand in hand with hyperæmia of the splanchnic area (chiefly the gut) is shown by the fact that if the mesenteric arteries are clamped, quite large doses of the venom do not produce any marked effect in the blood-pressure.

The paralytic action of the venom seems to be confined to the capillaries only. In the perfusion experiment it was observed that the veins and arteries are not dilated, on the other hand they show a tendency to constrict. The paralytic action of the venom on the capillaries was observed to be similar to that of histamine since the venom does not give any fall of blood-pressure after large doses of histamine and vice versa. Drugs like ether and chloroform which depress the capillaries, potentiate the action of the venom. Under the microscope, fine capillaries of the frog's omentum were seen to dilate widely when exposed to the action of daboia venom. Adrenalin and pituitrin, which tone up the capillaries and glucose, gelatine and gum-saline, which increase the total volume and the viscosity of the blood, tend to revive the blood-pressure. The hæmorrhagic tendency and enormous leakage of the plasma from the capillaries is further supported by the fact that the coagulation time is increased and the red cell count is also increased after large doses of the venom. From the above data we are justified in concluding that the venom has a paralytic action on the capillaries which increases the leakage, thus producing symptoms similar to that of shock. Death is secondary to shock and life can be saved if the shock can be overcome early.

Drugs like adrenalin and pituitrin which increase the tone of capillaries, glucose and gum saline which increase the total volume of circulating fluids, and cholesterol which prevents the hæmolysis of blood, are probably the favourable ones in the treatment of viper venom poisoning.

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ALANGIUM LAMARKII ITS CHEMISTRY AND PHARMACOLOGICAL ACTION

BY

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Alangium lamarkii, also known as *A hexapetalum* (Roxb), *A decapetalum* (Lam), and *A tomentosa*, is a small deciduous shrub belonging to the N O Cornacea. It grows widely in the tropical forests of Southern India and Burma. In vernacular it is called *ankola* (Sanskrit), *alola-dhera* (Hindi), *akarlan'a* or *baghan-kura* (Bengali), *onkila* (Gujrati) and *alang* or *ankoldmchettu* in Tamil and Telugu. The plant has alternate, petiolated, entire and three-veined leaves, and silky-white flowers. The fruit is like berries covered with a disc having an elongated calyx. The root is yellow in colour and has a closely grained texture. The bark has a cinnamon-brown colour, is corky in nature, and has a bitter taste.

The root-bark of this plant has long been used by the Hindu physicians as an alterative and emetic. In Sanskrit it is also called *Gupta-sneha* (the one of which the oil is hidden) and in the 'Nighantas' it is described as a bitter mucilaginous pungent drug which expels phlegm. It has been especially recommended in leprosy and syphilis and other skin diseases. According to Mohideen Sheriff (Watt, 1899) the drug, when administered in doses of 50 grains, acts as an emetic, while in 2 to 5 grain doses it acts as a diaphoretic and anti-pyretic. He further suggested that it could be safely used as a substitute for ipecacuanha. Dymock (1891) reports that an extract made from the root-bark is said to act as an anthelmintic and as a laxative, a decoction prepared from the bark and mixed with ghee is used in the treatment of snake-bite and rabies. The root-bark forms a soothing application for rheumatic joints. The fruit is believed to possess nutritive and anti-tubercular properties and is used in the treatment of phthisis. It is also a laxative and is used against internal piles.

CHEMICAL COMPOSITION

A systematic and thorough chemical analysis of the plant appears not to have been made. With a view to study in detail the chemical composition and pharmacological action of this reputed drug the present investigation was taken in hand. Several assays of the air-dried and powdered (60 mesh) root-bark obtained from different sources were made in the Department of Chemistry, and on the average showed the presence of 0.8 per cent alkaloidal base. For a systematic examination of the drug 50 g. of the powdered bark were extracted successively in a Soxhlet apparatus with petroleum ether (B.P. 35°C–60°C), ether (absolute), chloroform, absolute alcohol, 70 per cent alcohol and finally with water. The organic solvents were removed from each extract and the residues weighed. Petroleum ether extracted 0.39 per cent, ether 0.67 per cent, chloroform 0.63 per cent, absolute alcohol 3.6 per cent, 70 per cent alcohol 2.74 per cent, and water 2.43 per cent of the dry bark. In addition to alkaloidal base, some resins, fatty oils, tannins and potassium salts were found to be present.

Isolation and purification of the alkaloid—Two maunds of the air-dried bark was coarsely crushed and extracted in the cold with 92 per cent alcohol and the solvent removed under reduced pressure. The residue weighing 7 lb. 8 oz. was repeatedly treated with hydrochloric acid till completely exhausted. The solution was neutralized with 10 per cent sodium carbonate solution and a resinous precipitate, at the neutral point, was filtered and washed with a little water. The alkaloid was dissolved in 1 per cent hydrochloric acid solution, again neutralized and the precipitate at the neutral point separated. From the filtrate, the base was liberated with sodium carbonate solution and taken in chloroform. The base was extracted from chloroform with 1 per cent hydrochloric acid and again taken in chloroform. The solvent was dried over anhydrous sodium sulphate and the solvent removed. The dry alkaloid was extracted with warm benzene which removed the major portion of the highly coloured matter. The base was dissolved in the smallest quantity of chloroform and fractionally precipitated with petroleum ether. The precipitate at the second fraction, lemon-yellow in colour, was collected for further purification.

The coloured base was dissolved in 1 per cent hydrochloric acid and was partially precipitated with addition of salt. The solution was made alkaline with sodium bicarbonate solution, the base was taken in ether, the solvent removed and the base was obtained as a lemon-yellow amorphous powder, M.P. 80°C–82°C. Its optical rotation was $[\alpha]_D^{35} = -34.0^\circ$ in chloroform. The name 'Alangin' has been suggested for this alkaloid.

The basic property of the alkaloid is not very strong. It is soluble in most of the organic solvents, such as alcohol, benzene, chloroform, ether, acetone, acetic ether and fairly soluble in water. It is insoluble in petroleum ether. It gives positive test with Mayer's, Kraut's and Sonnenschein's reagents. Attempts to prepare crystalline salts with this alkaloid proved unsuccessful. The hydrochloride of the base was prepared by passing dry hydrochloric acid gas in a solution of the base in benzene.

Local action—The hydrochloride salt of this alkaloid is readily soluble in water and when dissolved in normal saline in concentration of 1 per cent, it yields a light yellowish-brown solution which is odourless and has an intensely bitter taste.

Applied locally to the intact skin it does not produce any signs of irritation or inflammation. A 2 per cent solution of this alkaloid instilled into the conjunctival sac of a cat or rabbit produced signs of a mild irritation in a short time. Within 24 hours the eyes get congested, oedematous and full of secretion. There was no anaesthesia of the cornea and the pupillary reflexes were not affected. When injected subcutaneously, the drug is not irritant and produces no marked signs of inflammation. When injected deep into the muscles the alkaloid is readily absorbed leaving no trace at the sight of injection after six hours.

Action on unicellular organism—Chopra and Chowhan (1931) have observed that whenever *Paramaecium caudatum* comes in contact with any poisonous or irritant substances, it shows the usual 'avoiding reaction' movements and there is a change in their co-ordination and power of swimming. Similar effects were observed whenever a fresh culture of the paramoecium was mixed with a solution of the alkaloid in concentration of 1 in 10,000 to 1 in 8,000. With still higher concentrations, e.g., 1 in 2,000 and more, there was immediate cessation of movements and death. The alkaloid is not very toxic to these ciliates.

Action on the alimentary system—As has been already referred to, *Alanguum lamarkii* has been used as a substitute for ipecacuanha. In our experiments it was observed that whenever a cat was fed with doses of 60 mg to 120 mg of the alkaloid through a stomach tube, there was a marked increase in the flow of thick and glaucous saliva in about 15 to 20 minutes, followed later on by nausea and occasional vomiting. With larger doses consciousness was lost and death supervened in a short time.

The effect on the peristaltic movements of the intestines was studied by means of Jackson's enterograph in cats, anaesthetized with chloralose. Five to ten mg of the drug injected intravenously, produced a well-marked increase in the tone and peristaltic movements. This is apparently a vagal effect as the extra tone is abolished by previous atropinization. The contractions of the gall-bladder are also stimulated.

Action on the respiratory system—The action of the drug on the respiratory mechanism was determined by studying its effect on the tracheal respiration and intra-pleural pressure. Urethane anaesthesia, sometimes supplemented with ether, was used. With 2 mg to 4 mg of the alkaloid the rate and the amplitude of the respiratory movements were increased while with larger doses, such as 6 mg to 8 mg, the initial stimulation was followed immediately by an irregular and spasmodic type of breathing. These effects were relieved both by the section of the vagi or by giving small doses of atropine. The respiratory centre has therefore some part to play in this stimulation. Injections of 2 mg to 4 mg of the drug produced a slight dilatation of the bronchi, but larger doses showed a decrease in the volume indicating contraction of the bronchioles and respiratory irregularities. The transitory dilatation produced is due to the stimulation of the respiratory centre and the decrease which follows is due to bronchial constriction produced by stimulation of the terminations of the vagi.

Action on the circulatory system—(a) *Blood-pressure*—One to two mg of the alkaloid when injected into the femoral vein of a cat under chloralose anaesthesia produced a slight initial rise in blood-pressure followed by a marked fall amounting to 20 mm of mercury. The blood-pressure curve gradually returned to normal level in a few minutes. With larger doses, e.g., 10 mg to 12 mg of the alkaloid, a

permanent and sustained fall of blood-pressure was produced which did not regain its normal level for a long time. Sometimes oscillations and irregularity in the blood-pressure curve were observed. The fall of blood-pressure may be attributed to one or more of the following factors —

- (1) The direct depressant action of the alkaloid on the vasomotor centre
- (2) The depression of the sympathetic or stimulation of the vagal nerve-endings
- (3) The depressant action on the cardiac muscles
- (4) The dilatation of the systemic blood vessels

These factors were further investigated in order to determine the real cause which is responsible for this fall in the blood-pressure. In a few cats the brain and the spinal cord were destroyed so as to eliminate the control of the medullary and spinal centres. In another series in addition to decerebration, the sympathetic nerve-endings were also put out of action by the administration of increasing doses of ergotoxin. In both the cases the usual fall of blood-pressure was obtained. This shows that neither the vasomotor centre nor the sympathetic nerve-endings play any important part in the fall of blood-pressure produced. If, however, the vagal nerve-endings were completely paralysed with atropine in a decerebrated animal, the administration of the alkaloid produced little or no effect on the blood-pressure. It would appear therefore that the fall in blood-pressure is mainly due to the stimulation of the vagal terminals.

(b) *Heart* —Cardiometer experiments showed a well-marked dilatation of the heart after administration of the alkaloid. An injection of 2 mg to 3 mg of the alkaloid produced a transitory acceleration of the beats of both the auricles and the ventricles in myocardiographic tracings. This stimulation is only transient and is followed by a definite depression soon after. If the dose is not too large, there is an attempt on the part of the circulatory system to re-adjust itself and the beats become more regular. With still larger doses the depression increased and the heart-beats often become irregular probably on account of the spasmodic type of respiration which was set up. This depression of the heart-beat was also observed after the vagal nerve-endings had been paralysed with atropine.

The isolated hearts of rabbits and kittens were perfused with oxygenated Locke's solution at pH 7.4 and temperature 37.5°C. With dilutions ranging from 1 in 500,000 to 1 in 200,000 the alkaloid produced a transitory stimulation followed later by depression and slowing of the beats. With higher concentrations, such as 1 in 50,000 to 1 in 10,000, there was no initial stimulation but depression was evident from the very beginning.

(c) *Volume of visceral organs* —With doses of 2 mg to 3 mg intravenously there was an increase in the volume of all the abdominal organs, the intestines, the spleen and the kidneys, corresponding to the fall in the systemic blood-pressure. This shows that there was a good deal of accumulation of blood in the splanchnic area.

(d) *Blood vessels* —The systemic blood vessels of a pithed frog were perfused with 0.7 per cent saline according to Trendelenburg's technique. The number of drops passing out in 30 seconds were recorded. The alkaloid was then added in concentration varying from 1 in 50,000 and upwards. No definite action was observed till the concentration reached 1 in 20,000. In such and stronger concentrations, there was a slight reduction in the number of the drops, showing that there

was some constriction of the blood vessels in the frog. In cats when 8 mg to 10 mg of the alkaloid were injected intravenously there was a slight increase in the volume of the limb. The limb vessels showed a mild constriction, while the mesenteric vessels showed dilatation when perfused with such dilutions as 1 in 200,000 to 1 in 100,000.

Toxicity—The toxicity of the alkaloid was determined on frogs, white mice, guinea-pigs and cats.

(a) *Frogs*—A 2 per cent solution of the drug was injected into the ventral lymph sac. The drug was found to be entirely non-toxic to amphibia in doses varying between 80 mg and 100 mg per kilogram. With slightly larger doses the animals appeared to be stunned and in half an hour their movements became very sluggish. The main feature observed was a marked moistening of the skin, profuse droplets of glandular secretion appearing all over the dorsal cutaneous surface of the body. After an hour the animal lay flat on its belly with legs extended and was unable to correct itself when turned on its back. The respiration became slow and shallow but complete recovery occurred in 48 to 72 hours.

(b) *White mice*—Forty to fifty mg of the alkaloid injected into the dorsal vein of tail produced instantaneous death. With smaller doses the following symptoms were observed. Marked restlessness, increased respiratory rate followed by gasping and ærophagy, complete paralysis of limbs occurred before death. Doses between 20 mg and 30 mg per kilogram produced death in about 18 to 24 hours.

(c) *Guinea-pigs*—The minimum lethal dose was found to be 70 mg per kilogram when injected intraperitoneally. With smaller doses the symptoms observed were similar to those produced in white mice.

(d) *Cats*—A 2 per cent solution of the alkaloid was introduced into the stomach of cats by means of a stomach tube. Twenty to forty mg per kilogram produced nausea, profuse salivation, severe retching and occasional vomiting, followed half an hour later by choreæform movements, dyspnoæic respiration and strychnine-like convulsions. Even after recovery which followed in about 2 hours, the animal exhibited inco-ordination of movements, rigidity and twitching of the extremities.

Post-mortem findings—In almost all cases the heart was found to be dilated and appeared to have stopped in diastole. Both the auricles and the right ventricle were full of blood, the lungs were pale and collapsed, the liver was congested and friable and the gall-bladder in all cases was distended and full of bile. The intestines were empty but were greatly congested and in a state of tonic contraction. The spleen and kidneys were slightly congested and the urinary bladder was empty.

(e) *Action on sweat glands*—It has been observed that the drug has a selective action on the para-sympathetic nerve-endings and when administered in large doses it acts as an emetic. In animals the salivary secretion is greatly increased under its effect. In the case of frogs a good deal of glandular secretion was seen on the dorsal surface after administration of the drug. The effect of the alkaloid was therefore tested on the sweat gland in cat's paws. Intravenous injection of 10 mg of the alkaloid produces little effect but after 30 mg a definite increase in the secretion of sweat was observed.

DISCUSSION

From the experimental data given above it will be observed that the alkaloid isolated from *Alangium lamarkii* produces a slow and maintained fall of blood pressure. In very small doses the alkaloid appears to have a mild stimulant action but in ordinary doses it is depressant to the heart. The respiratory movements are markedly increased followed later by irregular and spasmodic type of breathing. The intensity of these effects is considerably reduced after the vagi are cut or their terminations are paralysed with atropine. The volume of the abdominal organs is increased with the fall of blood-pressure showing that the blood accumulates in the splanchnics. The alkaloid also increases the tone of the intestinal musculature and stimulates the automatic movements of the spleen, these effects disappear after atropine. In feeding experiments it was observed that the animal retched a good deal immediately after the drug was introduced into the stomach. Larger doses produced breathlessness and ærophagy followed by inco-ordination of movements, tetaniform convulsions and unconsciousness.

A perusal of these results shows that the alkaloid isolated from *Alangium lamarkii* has a selective action on the para-sympathetic mechanism. The action is most marked on the gastro-intestinal tract. On the circulatory and respiratory systems, the action of the drug is much less in evidence. The medullary centres are probably stimulated, especially the vomiting centre. Sweat secretion is increased due to the stimulation of the para-sympathetic nerve-endings. Whether the drug has any action on the sweat glands themselves cannot be definitely stated at present.

SUMMARY AND CONCLUSIONS

(1) The active principle contained in *Alangium lamarkii* is an alkaloid to which the name of *alangin* is given.

(2) This alkaloid has a powerful stimulant action on the para-sympathetic mechanism.

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A PRELIMINARY NOTE ON THE PHARMACOLOGICAL ACTION OF *ANTIARIS TOXICARIA*

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Antiaris toxicaria (Upas tree), N O Urticaceæ, is known as *chandla*, *chandakuda*, *sapsundi* in Marhatti, *netavil*, *netta-vil maram* in Tamil, *netlavil* in Malayalam and *Hmyaseik*, *Myeh-seik* in Burmese. It is found in the Deccan Peninsula, and the Ghats from the Concan downwards. It is a large evergreen tree reaching the height of 250 feet. The following description from Chopra's 'Indigenous drugs of India' (1933) will give the ideas prevalent about this tree. 'The tree has become famous since the latter part of the eighteenth century as the source of a most deadly poison. Most exaggerated statements regarding this plant were circulated by a Dutch surgeon about that period. It was stated that all living things approaching within miles of these trees fall a victim to the effects of the poison exhaled from them. These are now universally recognized to be myths and not facts. The juice derived either from the leaves or the bark of the tree is nevertheless distinctly poisonous. The sap is of a dark-brown colour with a gummy consistency, bitter and biting in taste. It is used to this day as an arrow poison by the Karens in Java, Malaya and particularly in Burma where the tree is most commonly found. Its poisonous properties, however, are not widely known in the Deccan and Ceylon where also the tree is frequently met with. In the Concan and Canara the bitter seeds are used as a febrifuge and as a remedy in dysentery, one-third to one-half of a seed being given three times a day. In Travancore *A. toxicaria* is known as the 'sacking tree' and is not regarded by the people as poisonous, the same is the case in Coorg, where sacks and even garments are sometimes made from the inner bark.'

Chemical composition—A large amount of work has been done on the composition of the milky juice of this plant since 1838. The latest work by Kilham shows that the juice contains the following constituents: (1) antiarol, $C_9H_{12}O_4$, the trimethyl ether of 1, 2, 3, 5 phenetetrol, (2) potassium nitrate, in large amounts, (3) a crystalline resin, named antiarresin, $C_{39}H_{56}O_2$, which is the cinnamyl ester of α -amyrin, (4) a crystalline protein, (5) an acid, $C_{16}H_{14}O_7$ and (6) three active glucosides: (a) α -antiarin, $C_{27}H_{42}O_{10} \cdot 4H_2O$, crystalline, M.P. 220° to $225^\circ C$, (b) β -antiarin, $C_{27}H_{38}O_{10} \cdot 3H_2O$, crystalline, M.P. $206^\circ C$ to $207^\circ C$, and (c) γ -antiarin which is amorphous. These glucosides occur in varying amounts in different samples and are said to possess strong digitalis-like action on the heart.

Pharmacological action—Regnault in 1878 worked with a juice supposed to have been derived from *A. toxicaria* and used by the savages of Tonking to poison their arrows. He found that this substance had a powerful poisonous action on the heart. Boinot and Hedon also examined the brown latex used as an arrow poison and showed that 3 drops of a solution of 0.5 gramme of the poison in 10 grammes of water arrested the pulsation of a frog's heart in 7 minutes, when locally applied and that 100 mg per kilo of body-weight were rapidly fatal to animals. Very little work has been done during the last 30 years on the pharmacological action of this drug and hence a detailed study was undertaken. As the material available was not sufficient for the isolation of the individual glucosides, the action of the drug as a whole was investigated to begin with. Throughout this investigation either the aqueous or the alcoholic extract of the dried gum was used, care being taken to free them from any mechanical impurities as much as possible.

Cardiovascular system—Investigations on this system were carried out in cats, urethane or chloralose being used as an anæsthetic. In very small doses, e.g., 0.1 mg to 0.2 mg per kilo of body-weight, the drug had no appreciable effect on the blood-pressure. After administration of 0.75 mg to 1.25 mg per kilo of body-weight intravenously, effects were visible in a few minutes. The blood-pressure showed a slight but steady rise. After administration of slightly larger doses, e.g., 2 mg per kilo of body-weight of the extract, a rise of blood-pressure occurred starting almost immediately after and continuing for a long time. Irregularity of the blood-pressure curve was sometimes observed in particularly sensitive animals. Larger doses, e.g., 4 mg to 6 mg per kilo produced effects which were of great toxicological interest (Graph, fig. 1). If the injections were given slowly, the blood-pressure began to rise almost immediately and the rise was steady and persistent for about 10 minutes. Occasional irregularities in the blood-pressure curve were visible. This condition lasted for a few minutes and then the blood-pressure showed a decline followed by a temporary recovery. This state of affairs recurred several times, till finally in about half an hour the blood-pressure fell to a very low level. In many experiments the blood-pressure curve touched the base line, i.e., fell to zero for a few minutes and then it suddenly shot up again, sometimes to even above the normal and remained at that level for quite a long time. Such changes go on for some time before the animal dies. During these periods distinct fibrillations of the heart were visible in the blood-pressure curve. Sudden injection of a very large dose, e.g., 6 mg to 8 mg per kilo of body-weight, showed practically no rise of blood-pressure but a fall and death from collapse (Graph, fig. 1).

GRAPH

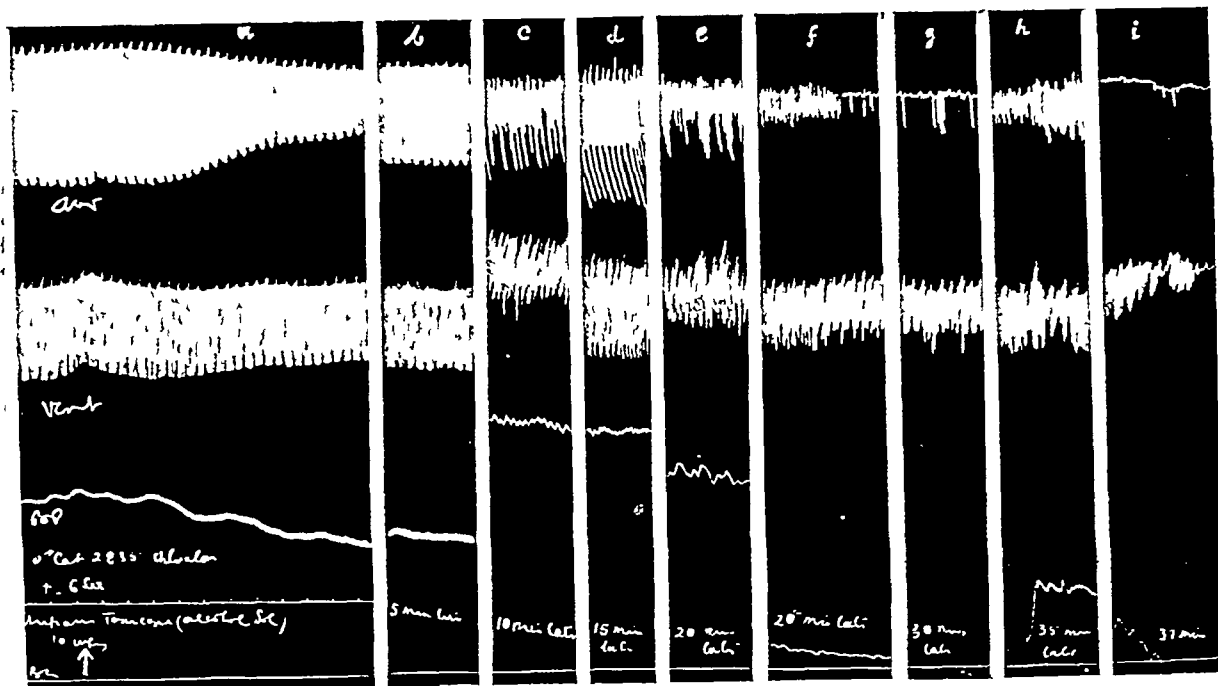


Fig 1

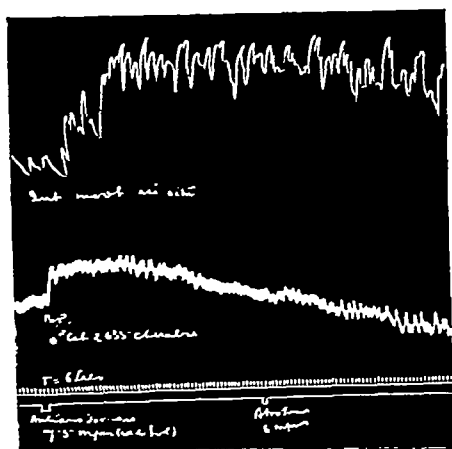


Fig 2

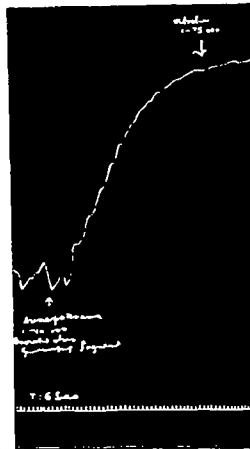


Fig 3

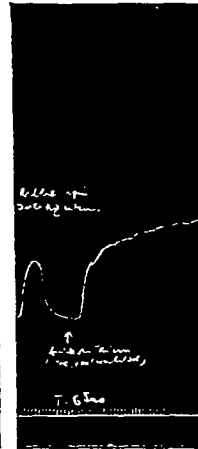


Fig 4

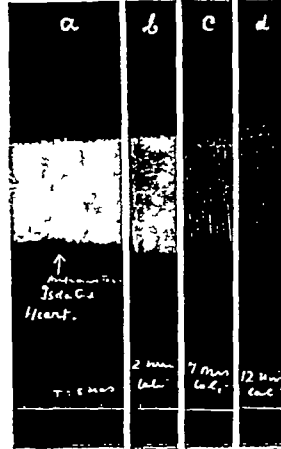


Fig 5

Fig 1, a, b, c, d, e, f, g, h and i: Male cat, 2,835 g, chloralose. Myocardiographic tracing and blood pressure 10 mg of alcohol soluble extract of *Antiaris toxicaria* injected into the femoral vein. Note slight rise of blood pressure immediately after injection followed by a fall (Fig 1, a and b). At c and d the blood pressure is at its highest level, and irregularity is noticeable. At f the blood pressure is almost zero and at g it has touched the base line. At h the tendency to readjustment of the circulation is evident. The auricular systole is weakened from the very beginning. At c and d the auricles show marked inhibition both in its systolic and diastolic phases. The ventricles have also followed suit. Missing of beats and irregularities of conduction are also evident. At f, g and i almost complete auricular paralysis is seen followed by irregular ventricular beats. Interval between the tracings a to h are 5 minutes, between h and i is 2 minutes. Time—6 seconds.

Fig 2: Male cat, 2,635 g, chloralose. 7.5 mg of water soluble extract of *Antiaris toxicaria* injected. Note stimulation of both tone and peristaltic movements of the intestine, not relieved by atropine. Time—6 seconds.

Figs 3 and 4: Isolated uterus of guinea pig (pregnant) and rabbit (virgin) perfused with 1 in 40,000 solution of *Antiaris toxicaria*. Note the marked increased tone in both. Time—6 seconds.

Fig 5: Isolated heart of kitten perfused with 1 in 100,000 solution of *Antiaris toxicaria*. The increase of amplitude of contraction and slowing of the heart are seen. The interval between the tracing a and b is 2 minutes and those between b, c and d are 5 minutes. Time—6 seconds.

All the above experiments were repeated in animals treated with large doses of atropine to paralyse the vagal endings. Similar results were obtained showing that the vagi did not play any part. It was further observed that if the animals were previously treated with nicotine or repeated doses of ergotoxin, to paralyse the ganglion cells and the sympathetic nerve-endings respectively, the effects of this drug were not appreciably altered. We are therefore led to believe that the drug acts mainly on the cardiac musculature.

Myocardiograph—Records of the movements of the auricles, the ventricles and the carotid blood-pressure were taken in chloralosed cats under artificial respiration (Graph, fig 1). It was observed that 1/10 mg to 1/5 mg per kilo of body-weight had little or no action on the beats of the auricles and the ventricles. Injections of about 0.75 mg to 1.25 mg per kilo showed a slight increase in the amplitude of the beats of both the auricles and the ventricles after an interval, slowing was slight and sometime absent. When 2 mg of the extract per kilo were injected, an exaggeration of the condition described above was observed. In some cases an appreciable slowing of the beats was produced, especially when the alcoholic extract was injected. As a rule fibrillations were not observed with this dose. When bigger doses, e.g., 4 mg to 6 mg per kilo of body-weight of the extract were given the initial increase in the amplitude of the auricles and the ventricles was absent, but on the other hand the force and frequency of the beats were considerably reduced and fibrillations of the auricles were observed in 5 to 8 minutes. With the onset of this fibrillation the blood-pressure curve showed a marked irregularity (Graph, fig 1). Within a few minutes this irregularity passed off and the heart once more started beating regularly. These alternate periods of irregularity and revival were repeated several times till the heart became very irregular and the blood-pressure fell to a very low level. Still later the auricular beats became very feeble and irregular as compared with those of the ventricle and finally the auricles appeared to stop beating altogether while the ventricles went on beating irregularly. In some cases an apparent revival of the semi-paralysed auricle was observed. In all cases the auricles stopped beating long before the ventricles.

In decerebrated animals similar effects were observed. Atropinized animals also showed the effects described above, except that no appreciable slowing was observed. This suggested that the drug possibly had some effect on the vagal nerve-endings.

Isolated heart—The isolated hearts of kittens when perfused with 1 in 100,000 to 1 in 40,000 solution of the watery extract of *Antiaris toxicaria* produced a slight but steady increase in the amplitude of the beat (Graph, fig 5), with larger doses the increase of amplitude became gradually less and less apparent. With still bigger doses the amplitude of the heart was markedly diminished from the beginning.

Continuous perfusion of the heart of a kitten with 1 in 100,000 solution of the drug showed, within a few minutes, a slight increase in the amplitude of the heart-beat, the frequency being slightly slowed. Attacks of arrhythmia lasting for a few minutes at a time followed by regular beating of the heart recurred at short intervals several times before the heart stopped in diastole. The auricles were distended with

blood The coronary outflow was diminished in practically every case especially when large doses were given

Blood vessels—Continuous perfusion of the vessels of the hind limbs and the mesenteric vessels with 1 in 100,000 solution of the water-soluble extract showed a gradual constriction of the vessels which was unaffected by ergotoxin It has already been pointed out that ergotoxin has no action on the pressor effect of the drug These facts show that the drug probably acts on the musculature of the blood vessels

Respiration—It was observed that within a few minutes of injection of 5 mg of this drug, the intratracheal pressure was diminished and the rate of respiration was slightly slowed This was soon followed by broncho-constriction which persisted for some time after the injection The respiration went on for quite a long time after the heart stopped beating.

Intestine—Perfusion of isolated intestine of kitten with 1 in 40,000 solution of drug showed a definite increase in the tone and peristaltic movements of the intestines The same effects were also observed when the intestine was previously perfused with atropine Although previous treatment with atropine did not alter the effect of the drug on the intestines, very small quantities of adrenalin completely prevented this action Experiments on the intestines *in situ* in chloralosed cats showed a marked increase in the tone of the intestine which was not relieved by administration of atropine (Graph, fig 2) These showed that increase of peristalsis was due neither to the stimulation of the parasympathetic nor to the inhibition of the sympathetics

Uterus—The uteri of guinea-pigs and rabbits showed tonic contraction when perfused with this drug (Graph, figs 3 and 4) in concentrations of 1 in 100,000 to 1 in 40,000 This effect was not altered by the administration of atropine and was probably produced by direct action on the musculature, because if the effects were due to the stimulation of the sympathetic endings, the uterus of the guinea-pig would have responded by relaxation instead of contraction

Toxicity—Intravenous injection of 7.5 mg per kilo of water-soluble extract to cats proved fatal in about half an hour The respiration steadily became slower and deeper at first and later spasmodic

Guinea-pigs receiving 40 mg per kilo by subcutaneous injection died within 30 minutes to one hour After injection of fatal doses the animals showed signs of weakness of the hind limbs within a few minutes and were inclined to lie either on the side or on the abdomen Soon the respiration became rapid and occasional spasms of the voluntary muscles were observed The fore limbs showed signs of paralysis and the animal was unable to move, the respiration at this stage became deeper and laboured The frequency of the spasms increased and involuntary evacuation of urine and faeces was not uncommon at this stage Similar doses of the alcohol-soluble portion appeared to be much more toxic and produced death in a much shorter time

SUMMARY.

From the experimental data it is obvious that the dried juice from *Antiaris toxicaria* has a slight stimulant action on the heart and circulation In larger

does it acts as a strong cardiac poison. The action on the auricle is much stronger than that on the ventricles. The drug acts mainly on the myocardium. It may also have some effect on the terminations of the vagi as it was observed that administration of atropine before the drug reduced its slowing effect on the heart. It has no action on the higher centres because the drug acts equally well both in intact and decerebrated animals. The drug produces a marked tonic contraction of the isolated as well as the intact intestines and uterus of animals.

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THE EFFECT OF HEXYLRESORCINOL ON CATS

BY

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AND

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WHEN carbon tetrachloride was first introduced for the treatment of hookworm infection in man the early reports on its pharmacological effect on animals came from America. In these experiments dogs were mainly used although cats were also said to be tolerant of the drug in large doses. Chopra and Chandler (1925), however, found that cats in Calcutta were very susceptible to this drug and that ninety-two per cent of eighty-seven cats employed died from its effects, irrespective of the size of the dose or the diet employed. Hexylresorcinol was first recommended as an anthelmintic by Lamson, Ward and Brown (1930), and in their paper they stated that the drug produced reddening of the gastric mucosa, submucous hæmorrhage and even epithelial necrosis in dogs, and that the effects were increased by the addition of alcohol. On account of the above discrepancy in the findings of the American workers and of Chopra and Chandler in respect of carbon tetrachloride we decided to test hexylresorcinol to ascertain if it also had toxic effects on Calcutta cats.

Altogether twenty-four cats were employed, in twelve the drug was given in varying doses dissolved in alcohol, and in the other twelve it was dissolved in olive oil. In the series of cats in which the drug was dissolved in alcohol doses of 0.5 grain and 0.75 grain per kilogram of body-weight produced no signs or symptoms, in doses of 1.6 grains to 3 grains per kilogram all the cats treated showed drowsiness, and unsteadiness of the hind limbs in degrees of severity varying with the size of dose of hexylresorcinol, but they all recovered within twenty-four hours. Cats given four to five grains per kilogram all died within twenty-four hours, first becoming unconscious within six hours of the drug being administered.

When dissolved in olive oil hexylresorcinol was not quite so toxic, because it was found that although three cats died after doses of five, six and eight grains per

kilogram respectively two cats recovered after doses of six grains and seven grains, but they exhibited severe toxic symptoms

On account of the rapidity with which post-mortem changes occur in this climate, and because the cats practically all died during the night, it was impossible to examine them immediately after death, so a number of animals were given hexylresorcinol both in alcohol and olive oil in doses varying between one gram and six grains per kilogram and they were killed at times ranging between five and twenty-one hours after being given the drug

Dr M N De, Professor of Pathology of the Medical College, Calcutta, kindly examined microscopic sections from all these cats and gave us a detailed report from which the following summary has been taken —

Cats given hexylresorcinol in alcohol .

One grain per kilogram had no apparent effect on the organs examined

Two and a half grains to five grains per kilogram of body-weight had the following effects—

Stomach —Superficial necrosis with smaller doses, and in larger doses coagulation necrosis extending in places through half the depth of the mucosa, with congestion and occasional hæmorrhage or leucocytic infiltration

Duodenum —Doses of two and a half grains produced hypersecretion only, and five grains caused loss of epithelium in patches, together with some degree of congestion

Liver —Moderate congestion with some slight damage to the liver-cells. But one cat which received the maximum dose of five grains showed a normal liver

Kidneys —Moderate congestion going on to slight granular and fatty changes when the larger doses were reached

Cats given hexylresorcinol in olive oil .

Stomach —The damage was limited to congestion even when as much as six grains per kilogram of body-weight was given

Duodenum —Similar to stomach

Liver —Congestion with a certain amount of fatty changes and round-celled infiltration

Kidneys —Similar to those of cats which were given the drug in alcohol

These results show that the action of hexylresorcinol on the mucous membrane of the stomach and duodenum is much less severe when it is given in olive oil than when given in alcohol, but after its absorption the effect on the liver and kidneys appears to be about the same whichever vehicle has been employed. These findings do not agree with those of Lamson and Ward (1932) as the following quotation will show. 'Very careful microscopic examination of the various organs of the body after single large doses of hexylresorcinol and even after repeated administrations

over long periods failed to show in Leonard's experiments or in our own any pathological lesions' But this statement cannot be reconciled with that of Lamson, Ward and Brown (*loc cit*) that hexylresorcinol produces reddening of the gastric mucosa, submucous hæmorrhage and even epithelial necrosis in dogs. The latter finding is much more in agreement with ours than the former, and therefore we are inclined to consider it the more correct of the two.

The dose we have employed in cats is of course many times that given to man, but this was done deliberately to test the contention that hexylresorcinol was free from all toxic effect, and we consider our findings show this statement is not correct. It is true that with a dose of one grain per kilogram of body-weight for cats, which is approximately four times the maximum dose recommended for man, no pathological changes were found, so the drug is not considered dangerous to man under ordinary conditions. This is evident from the numerous reports that have already been published of its use on a fairly large scale, without any untoward symptoms occurring, as it has been given to several thousands of people in doses ranging between one and two grammes. At the same time we feel that our results on cats indicate that there is a distinct possibility of causing severe damage and illness in predisposed persons, whether from idiosyncrasy or intercurrent disease, and it should not be ignored. From our very limited experience of the use of this drug we can quote the following illustrative experience. Since the paper by Maplestone and Mukerji (1932) appeared we tried this drug, incidentally without any anthelmintic effect, on a patient with strongyloides infection accompanied by a certain amount of 'indigestion'. The patient although not in any sense endangered from the point of view of loss of life suffered considerable pain and exacerbation of his digestive disturbance, with consequent loss of appetite for some days after his treatment with hexylresorcinol. Apparently the drug considerably increased the already present irritation of the gastric and duodenal mucosa.

We therefore are of the opinion that unless a certain amount of care is exercised in selecting persons as suitable for treatment with hexylresorcinol accidents are liable to occur.

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BED BUGS IN RAREFIED AIR

BY

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IN connection with the problem of bed bugs (*Cimex hemiptera*) in railway carriages in India I was asked by Dr R J Dyson, Chief Medical Officer M and S M Railway, to provide some experimental evidence as to whether bugs and their eggs would be destroyed by a reduction in air pressure

There appear to be but few references to this subject in the literature. It is a recognized method of dealing with certain infected materials, such as books and furs, to put them in a suitable container, pass in steam, induce a partial vacuum and then admit formaldehyde. There is a suggestion that certain eggs, for example those of the book louse, would be damaged in such a partial vacuum. It has been stated that fleas, probably *Pulex irritans*, burst noisily when taken 10,000 feet up the Andes, but that bugs are not thuswise affected (Manson-Bahr, 1927).

Various preliminary experiments showed that bugs remained active when the air pressure was reduced by about half. Finally a collection of 35 bed bugs, including various larval instars, was placed in a container and the pressure was rapidly reduced until it was equivalent to only one inch of mercury. Twenty-four hours later at least 25 individuals were still active, the vacuum having been maintained. It had been expected that the creatures would probably not suffer from oxygen deficiency but that they would be damaged by rarefaction of air, especially by a somewhat sudden reduction of barometric pressure. A number of bug eggs was treated to the same degree of partial vacuum for ten minutes and some of these eggs afterwards hatched. It was noticed that in some cases the operculum of the egg was forced off.

Bed bugs and their eggs are not therefore destroyed by a degree of partial vacuum much greater than could be readily obtained in the case of an infested railway carriage. The same idea must have occurred to others, hence this note.

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EIJKMAN'S TEST ON WATER-SUPPLIES IN THE MADRAS PRESIDENCY

BY

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[Received for publication, August 22, 1933]

EIJKMAN (1904) produced evidence that in Holland very pure waters showed no organisms capable of fermenting glucose at 46°C even when quantities of 300 c c were tested. On the other hand the greater the evidence of probable pollution of a water, the smaller the quantity of that water which produced fermentation of glucose at 46°C. The only standard suggested by Eijkman referred to sand-filtered water. He considered that not more than two out of ten 10-c c quantities should produce fermentation of glucose at 46°C.

A number of investigators in different countries have subsequently studied Eijkman's methods. Among the more recent papers on this subject is that of Leiter (1929) who working in America came to the following important conclusions —

(1) That the *coli* group of organisms can be isolated in pure or almost pure culture in 24 hours at 46°C and that the *aerogenes* group is inhibited at that temperature.

(2) That a positive Eijkman-test is closely correlated with indole production and also with non-utilization of citrate and non-utilization of uric acid.

(3) That the Eijkman-test is uniformly positive for *coli* isolated from the faeces of human beings and warm-blooded animals and uniformly negative for *coli* from cold-blooded animals.

Brown and Skinner (1930) from their work in Minneapolis agreed that pure waters rarely give a positive Eijkman-test. They found, however, that the test failed to discriminate between samples giving positive presumptive tests (by standard

methods) due to the presence of *coli* and *aerogenes* respectively, because some faecal *coli* did not ferment glucose at 46°C while some strains of *aerogenes* succeeded

Ruchhoft and others (1931) in their exhaustive study of water bacteriology devoted but little attention to the Eijkman-test and their conclusions regarding its value appear to be much the same as those of Brown and Skinner. There is further evidence from Lisbon that *coli* strains from cold-blooded animals rarely ferment glucose at 46°C (two out of 65) while most strains of *coli* from warm-blooded animals give a positive Eijkman-test (de Megalhães, 1932)

As regards tropical water-supplies the Eijkman-test has not apparently been applied by many workers. In two recent important contributions to the bacteriology of tropical waters (Pawan, 1931, Hirst, 1932) the Eijkman-test is not mentioned. Taylor and Goyle (1931) applied the test to Rangoon waters and were favourably impressed by its significance. They suggested as tentative standards for tropical waters that samples from tube-wells should not give a positive Eijkman test in quantities less than 100 c c and that samples of surface waters and from shallow wells should give negative results in quantities less than 50 c c. Some experiments with Eijkman's test are also reported from Kuala Lumpur (Kingsbury, 1932). The results suggest that the test does not distinguish positive presumptive tests due to the presence of *coli* and *aerogenes* respectively but that it may help to separate certain samples where the acceptance of the lactose gas line or the 'lactose plus, indole plus' test would demand an unduly high standard.

We have carried out the Eijkman-test on a number of samples of Madras waters which we have at the same time examined by the usual routine methods. Having had some difficulty in understanding the exact procedure carried out by certain writers we give the methods adopted in some detail.

The water is added to MacConkey's bile-salt lactose broth and to Eijkman medium, the strength of the media and the quantities of water being shown in the following tables —

Details of cultures in MacConkey-medium

Number of dilution	Strength of MacConkey medium	Amount of medium c c	Water added c c	Number of tubes.
A	<div> <div>6 per cent peptone</div> <div>15 per cent sodi taurocholate</div> <div>15 " lactose</div> <div>Neutral red</div> <div>Reaction alkaline</div> </div>	7	20	1
B	50 per cent of A	7	10	2
C	50 per cent of A	5	5	3
D	33 per cent of A	3	<div>1</div> <div>0 1</div> <div>0 01</div>	<div>3</div> <div>3</div> <div>3</div>

Details of cultures in Eijkman-medium

Number of dilution	Strength of Eijkman medium	Amount of medium c c	Water added c c	Number of tubes
A	10 per cent glucose 10 " peptone 5 " NaCl Andrade indicator pH 7.2	8	50	1
B	50 per cent of A	4	10	1
C	10 per cent of A	5 2.5	5 1	1 1

The MacConkey-tubes are incubated at 37°C and the Eijkman-tubes at 46°C and they are all examined for the presence of acid and gas after 24 and 48 hours. At the end of the first 24 hours the tube with the second smallest amount of water showing acid and gas is selected (or the tube with the largest amount of water if no other is positive or if none of the tubes are positive). Dilutions from these tubes are sub-cultured on MacConkey-agar for discrete colonies which are selected and identified in the usual manner.

The samples are grouped according to the source and the results of the tests are tabulated for reference. The gas readings are those at 48 hours. Six of the ten well-waters and three of the ten river-waters gave a positive Eijkman-test while with lake-water and infiltration gallery-water the test was uniformly negative. There is thus some general agreement between the probable quality of the water and the result of the Eijkman-test.

Quite frequently, however, samples showing lactose fermenters at 37°C in small quantities of the water gave no fermentation of glucose at 46°C in amounts up to 50 c c. In most of these cases the lactose fermenters were shown to include true *coli* (M R + Indole + Citrate —) and sometimes these included organisms of Clemesha's group I. Thus, samples B1, B5, B7, C4, D2 and E5 showed lactose fermenters including Clemesha's group I, viz., *Schæffer*, in 0.1, 0.1, 1.0, 1.0, 5 and 5 c c respectively but the Eijkman-test was negative in quantities up to 50 c c. Only one of these, C4, showed *coli* (*neapolitanus*) in sub-cultures from the negative 50 c c Eijkman-tube. The first and third of these samples came from sources exposed to obvious faecal pollution. Out of the sixty samples there were eight which showed true *coli* in 0.1 c c or less but which gave no fermentation of glucose at 46°C. The test failed to demonstrate the inefficiency of the sand-filtration in the case of E5.

Sub-cultures from the Eijkman-tubes which showed no gas but merely turbidity and occasionally acidity, frequently revealed the presence of lactose fermenters. In only three cases were true *coli* isolated from the Eijkman-culture tubes when the corresponding lactose-tubes yielded no true *coli*. Two of these were samples of

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lake-water showing no lactose fermenters in 60 c c and no lactose fermenters in sub-cultures from the negative MacConkey-tubes. In the third case, B2, the unusual finding was confirmed by following up further selected colonies from the plates and the result was similar, viz, *lactis aerogenes* and *cloacæ* from the lactose-tube and *schæfferi*, *coscoroba*, *acidæ lactici* and *vesiculosus* from the Eijkman-tube.

In two cases—A3 and D3—the only lactose fermenters isolated from the Eijkman-tube were *aerogenes*, while the corresponding plates from the lactose-tubes showed both *coli* and *aerogenes*.

There is one doubtful instance of a false positive, viz, A7, in which case, however, no lactose fermenters were isolated from either the Eijkman- or the MacConkey-cultures. In every other case where the Eijkman-tube was positive there was corroborative evidence that the sample was not satisfactory, inasmuch as lactose fermenters were present in small quantities of the water and with one exception—A6—these were shown to include true *coli*.

A table is compiled showing how often *coli* and *aerogenes* were recovered from the MacConkey- and Eijkman-cultures respectively, and also the occurrence of the more common species of these groups. The figures suggest that the growth of *aerogenes* is inhibited in Eijkman-medium at 46°C to a greater extent than the growth of *coli*. As regards *coli* there was frequent failure to recover three of the four commonest species, and as regards *aerogenes*, No 73 and *cloacæ* at least were sometimes demonstrated in the Eijkman-cultures.

Frequency of certain organisms in sixty samples of water

	<i>coli</i> , all species	<i>vesiculosus</i>	<i>schæfferi</i>	<i>neapolitanus</i>	<i>coscoroba</i>
From MacConkey medium at 37°C	37	8	10	16	12
From Eijkman-medium at 46°C	22	8	5	12	8

	<i>aerogenes</i> all species	No 67	No 73	<i>lactis aerogenes</i>	<i>cloacæ</i>
From MacConkey medium at 37°C	13	5	2	7	9
From Eijkman-medium at 46°C	4	0	2	0	3

The actual numbers of the various species of organisms identified in the confirmatory tests are omitted from the main tables as they cannot be claimed to bear any relationship to the proportions in the original sample

The Eijkman-test, taken as the production of acid and gas in Eijkman's medium at 46°C, fails in some cases where the sample must be condemned from a knowledge of local conditions and the results of other tests. Sub-cultures from the Eijkman-tubes may fail to demonstrate organisms which are considered indicative of faecal pollution and which are present in very small quantities of water. The test carried out in this way presents false negatives, that is, it is not sufficiently sensitive, and it appears to add no useful information to the results of the usual routine tests

A modification of the Eijkman-test has recently been tried by Williams *et al* (1933). Their medium contained less carbohydrate and they claimed that there was less acid production and that all the true *coli* survived. They used a pH of 6.6 for all their presumptive test media. False positives due to either *sporebearers* or the *aerogenes* group were still troublesome however.

The presence of streptococci is also noted in the tables. In most cases these were heat-resistant. This point is of some interest and should be further investigated. Streptococci were far more often noted in the sub-cultures from the Eijkman-tubes than in those corresponding to the MacConkey-broth in which medium the growth of streptococci is acknowledged to be somewhat inhibited.

The use of a plain lactose-agar, without bile-salt, for sub-cultures from the Eijkman-tubes resulted usually in an overgrowth of Gram-positive bacilli which obscured both coliform bacilli and streptococci.

SUMMARY

The Eijkman-test has been found unreliable for Madras waters. For example, a water from a source exposed to pollution and showing true *coli* in very small quantities, e.g., 0.1 c.c., may give a negative Eijkman-test with amounts up to 50 c.c.

It is shown that there is frequent failure to recover true *coli* after 24 hours' culture in Eijkman's medium at 46°C, while *aerogenes* sometimes survives under these conditions.

It appears that streptococci are commoner in tropical waters than has been supposed.

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A Well-waters

Number.	Lactose fermentors present in c c	Colonies from lactose tube	Eijkman test positive in c c	Colonies from glucose tube	Strepto cocci
A1	0 1	<i>coli</i> <i>acidi lactici</i> <i>neapolitanus</i>	5	No 1 f	—
A2	0 1	<i>coli</i> <i>acidi lactici</i>	50 negative	No 1 f	—
A3	0 1	<i>coli</i> <i>r pneumoniae</i> No 74 <i>aerogenes</i> No 67 No 73	50 „	<i>aerogenes</i> No 73	—
A4	5	<i>coli</i> <i>coscoroba</i>	50 „	No 1 f	—
A5	0 1	<i>coli</i> <i>schafferi</i> <i>neapolitanus</i> <i>aerogenes</i> No 98 <i>lactis aëro</i> <i>cloacæ</i>	50	No 1 f	+
A6	0 1	<i>aerogenes</i> No 67 <i>cloacæ</i>	10	No 1 f	+
A7	60 negative	No 1 f	50	No 1 f	—
A8	0 01	<i>coli</i> <i>coscoroba</i> <i>aerogenes</i> No 73 <i>lactis aëro</i> <i>cloacæ</i>	50 negative	<i>coli</i> No 66 <i>aerogenes</i> <i>cloacæ</i>	—
A9	0 1	<i>coli</i> <i>coli mutab</i> <i>coscoroba</i>	5	<i>coli</i> <i>vesiculosus</i> <i>schafferi</i> <i>neapolitanus</i> <i>coscoroba</i>	+
A10	1 0	<i>coli</i> <i>neapolitanus</i> No 76 <i>coscoroba</i>	5	<i>coli</i> <i>vesiculosus</i> No 71 <i>neapolitanus</i> <i>coscoroba</i>	+

Note — Nos 5 and 7 have covers and pumps
 Nos 1, 4 and 10 have pumps
 The others may be fouled by buckets and ropes

B River-waters

Number	Lacto-fermenters present in c c	Colonies from lactose tube	Eijkman test positive in c c	Colonies from glucose tube	Streptococci
B1	0.1	<i>coli</i> <i>schæfferi</i> <i>neapolitanus</i>	50 negative	No 1 f	+
B2	0.01	<i>aerogenes</i> <i>lactis aerog</i> <i>cloacæ</i>	50 „	<i>coli</i> <i>schæfferi</i> <i>coscoroba</i>	—
B3	0.01	<i>coli</i> <i>coscoroba</i> <i>aerogenes</i> No 67 <i>lactis aerog</i> <i>cloacæ</i>	30 „	<i>coli</i> <i>vesiculosus</i> <i>neapolitanus</i>	—
B4	1.0	<i>coli</i> <i>vesiculosus</i> <i>neapolitanus</i> <i>coscoroba</i>	50 „	No 1 f	—
B5	0.1	<i>coli</i> <i>schæfferi</i> <i>neapolitanus</i> No 109	50 „	No 1 f	+
B6	0.01	<i>coli</i> <i>vesiculosus</i> <i>r. pneumoniae</i> <i>gasiformans</i>	50 „	<i>coli</i> <i>vesiculosus</i> No 40 No 109 <i>aerogenes</i> No 73 <i>cloacæ</i>	+
B7	1.0	<i>coli</i> <i>schæfferi</i> <i>neapolitanus</i> <i>coscoroba</i>	50 „	No 1 f	—
B8	0.1	<i>coli</i> <i>vesiculosus</i> <i>schæfferi</i> <i>coscoroba</i>	5	<i>coli</i> <i>vesiculosus</i> No 40 <i>neapolitanus</i> <i>coscoroba</i>	+
B9	5	<i>coli</i> <i>neapolitanus</i>	50	<i>coli</i> <i>coscoroba</i>	—
B10	1.0	<i>coli</i> <i>schæfferi</i> <i>neapolitanus</i>	50	<i>coli</i> <i>coscoroba</i>	—

Note — Nos 1, 7 and 8 are grossly polluted by bathers, etc
 Nos 2 and 6 are stagnant and No 10 is in flood
 Nos 3, 4 and 5 are fairly clear running streams

C. Infiltration gallery-water.

Number	Lactose fermenters present in c c	Colonies from lactose tube	Eijkman-test positive in c c	Colonies from glucose tube	Strepto cocci
C1	10	<i>coli vesiculosus</i>	50 negative	<i>coli vesiculosus</i>	+
C2	80	No l f	50 „	No l f	—
C3	5	<i>aerogenes lactis aerog</i>	50 „	No l f	+
C4	10	<i>coli schæfferi neapolitanus.</i>	50 „	<i>coli neapolitanus</i>	+
C5	20	<i>coli neapolitanus</i>	50 „	<i>coli neapolitanus</i>	—
C6	20	<i>coli vesiculosus</i>	50 „	No l f	—
C7	60	No l f	50 „	No l f	—
C8	20	<i>coli neapolitanus</i>	50 „	No l f	—
C9	60 negative	No l f	50 „	No l f	—
C10	5	<i>coli neapolitanus.</i>	50 „	<i>coli schæfferi neapolitanus</i>	—

Note — Nos 5, 9 and 10 are in the bank of impounding reservoirs
The others are in the banks or beds of rivers which are dry during most of the year.

D Impounding reservoir-water

Number	Lactoso fermenters present in c c	Colonies from lactose tube	Eijkman + test positive in c c	Colonies from glucose tube	Strepto cocci
D1	5	<i>coli coscoroba</i>	50 negative	No l f	—
D2	5	<i>coli schæfferi</i> <i>aerogenes cloacæ</i>	50 „	No l f	—
D3	0 001	<i>coli r pneumoniae</i> No 74 <i>gasiformans</i> <i>aerogenes lactis aerog</i>	50 „	<i>aerogenes cloacæ</i>	+
D4	10	<i>aerogenes</i> No 67 <i>lactis aerog</i>	50 „	No l f	—
D5	1	<i>coli coscoroba</i>	10	<i>coli neapolitanus</i> <i>coscoroba</i>	+
D6	5	<i>aerogenes cloacæ</i>	50 negative	No l f	—
D7	10	<i>coli vesiculosus</i> <i>acidus lactici</i>	50 „	<i>coli schæfferi</i> <i>neapolitanus</i>	+
D8	0 1	<i>coli r pneumoniae</i> <i>gasiformans</i> <i>aerogenes</i> No 67 <i>cloacæ</i>	1	<i>coli acidus lactici</i> <i>vesiculosus</i> No 101	+
D9	5	<i>coli vesiculosus</i> <i>neapolitanus</i>	50	<i>coli neapolitanus</i>	—
D10	5	<i>coli schæfferi</i>	50 negative	<i>coli schæfferi</i> <i>neapolitanus</i>	+

Note — Nos 2, 4, 9 and 10 appear fairly well protected

The others are open to pollution from cattle or bathers or by inflowing surface water near habitations

534 *Eijkman's Test on Water-Supplies in the Madras Presidency**E Treated waters*

Number	Lactose fermenters present in c c	Colonies from lactose tube	Eijkman test positive in c c	Colonies from glucose tube	Strepto cocci
E1	60 negative	No l f	50 negative	No l f	—
E2	60	<i>arogenes</i> <i>cloacæ</i>	50 „	No l f	—
E3	60 negative	No l f	50 „	No l f	—
E4	10	<i>coli</i> <i>gasiformans</i>	50 „	<i>coli</i> No 109	—
E5	5	<i>coli</i> <i>schæfferi</i> <i>neapolitanus</i> <i>coscoroba</i> No 109	50 „	No l f	+
E6	60	<i>coli</i> <i>acidæ lactici</i>	50 „	No l f	—
E7	60 negative	No l f	50 „	No l f	—
E8	60 „	No l f	50 „	No l f	—
E9	60 „	No l f	50 „	No l f	—
E10	5	<i>coli</i> <i>acidæ lactici</i> <i>vesiculosus</i>	10	<i>coli</i> <i>acidæ lactici</i> No 101	+

Note—Nos 3, 6 and 8 are after alum treatment and mechanical filtration The others are after slow sand filtration

F Red Hills lake-water

Number	Lactose fermenters present in c c	Colonies from lactose tube	Eijkman-test positive in c c	Colonies from glucose tube	Strepto cocci
F1a	60 negative	No l f	50 negative	No l f	—
F1b	60 „	No l f	50 „	<i>coli</i> <i>vesiculosus</i>	—
F2a	60 „	No l f	50 „	No l f	—
F2b	60 „	No l f	50 „	No l f	—
F3a	60	<i>coli</i> <i>coscoroba</i>	50 „	No l f	—
F3b	60 negative	No l f	50 „	<i>coli</i> <i>neapolitanus</i> <i>coscoroba</i>	—
F4a	60 „	No l f	50 „	No l f	—
F4b	60 „	No l f	50 „	No l f	—
F5a	60 „	No l f	50 „	No l f	—
F5b	60	<i>coli</i> <i>neapolitanus</i>	50 „	No l f	—

Note—These samples are from different parts of a very large impounding reservoir at least half a mile from the edge and taken at depths of three and five feet respectively

The few intermediaries with the reactions M R + Citrate + have been included in the *coli* group, viz, *r pneumonia*, *gasiformans* and No 109.

BIOLOGICAL AND COLORIMETRIC ASSAY OF VITAMIN A IN SOME INDIAN FRESH-WATER FISH OILS

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ALTHOUGH the fresh-water fish plays a very large part in the diet of a large section of the population of this country, yet very little is known about their relative nutritive value. The only investigation so far on record is that of Banerjee and Nag (1933) who tested a few samples of liver oils colorimetrically with antimony trichloride. The present investigation was taken up to determine the vitamin A content of the body oils of *Labeo rohita* (ru, rohit, ruhee), *Cirrhina mrigala* (mrigal, mrigal), *Clupea ilisha* (hilsa, eelish), *Catla buehanani* (katol, katla) and dham.

The above-mentioned fishes are found abundantly in the rivers of Bengal, Assam and Burma as also in other parts of the country, and are extensively used for edible purposes. '*Labeo rohu* is the best of all fresh-water fishes, sweet to the taste but slightly bitter, increases vitality. It contains nitrogen 17.5 per cent, fat 16.4 per cent, and salt 2.36 per cent' (Nadkarni, K. M. 'The Indian Materia Medica', pp 1130-1131). Hilsa and dham are sweet to the taste probably owing to the large amount of fat which varies from 20 to 25 per cent of the total body-weight. Mirgal is not so popular as the others particularly in Assam where many people do not take it in the belief that, if taken in excess, it gives rise to hysteria and epilepsy.

Various colour reactions have been proposed for the determination of vitamin A but the one proposed by Rosenheim and Drummond (1925) and modified by Carr and Price (1926) is considered to be specific for vitamin A. Ahmad and Drummond (1930) found that the results of animal test agree with those of colorimetric methods.

On the other hand some investigators are of opinion that there is no strict relation between colour reaction and biological assay of vitamin A. In view of the above, we studied the vitamin A contents of the different oils by both biological and colorimetric methods.

EXPERIMENTAL

The body oils of 'ruhee', 'margal', 'dhain' and 'hilsa' were prepared by grinding the flesh with anhydrous sodium sulphate and extracting with ether in an atmosphere of nitrogen. The ether was removed in an atmosphere of nitrogen and the oils were kept in coloured bottles in which the air was replaced by inert gas. The liver oil of 'katol' was kindly supplied to us by Dr K. P. Bose. The livers were treated with steam and the liberated oil was filtered through anhydrous sodium sulphate.

Young rats weighing at an average 40 g to 45 g were put on a vitamin A-free basal diet consisting of—

Purified casein	20 parts
Purified rice starch	71 „
Salt mixture (McCollum)	4 „
Dried brewer's yeast	5 „

The casein used was the B. D. H. light white product which was repeatedly extracted with fresh quantities of boiling alcohol and finally with ether. Each of the rats received one drop of Radiostol (B. D. H.) twice weekly throughout the preparatory and the testing period to supply the antirachitic factor.

The majority of rats showed steady weight after 4 or 5 weeks on the basal diet. About 80 per cent of them developed symptoms of xerophthalmia and 3 per cent ear-sore with bleeding. They were then given measured supplements of different oils in doses of 100 mg, 50 mg and 20 mg. Olive oil was used for dilution. In order to compare the vitamin A values of the fresh-water fish oils with a standard cod-liver oil, a set of rats were tried with L. de Jongh cod-liver oil in doses of 20 mg. The oils were directly administered into the mouth of the rats before giving them the basal diet. The test with the supplements were carried out for 4 to 5 weeks but in the case of the rats which showed little or no growth the supplement was changed to an adequate dose of another oil in order to demonstrate that the animals were still capable of growing.

The body oil of hilsa and the liver oil of katol were tried on ten rats in doses of 100 mg. None of the rats showed any tendency to grow but remained constant in weight for nearly 15 days and then began to decline. Rats No. 46 and No. 48 (Fig. 1) were given a changed supplement of 20 mg of margal-body oil, rats No. 45 and No. 50 were changed to a supplement of 20 mg of ruhee-body oil. The rats showed prompt response in growth. The eye disease was cured and the yellow pigmented colour of the rats changed in a remarkable manner to glossy milk-white appearance. The animals whose supplement was not changed died after a few days as instanced by that of rat No. 47 (Fig. 1).

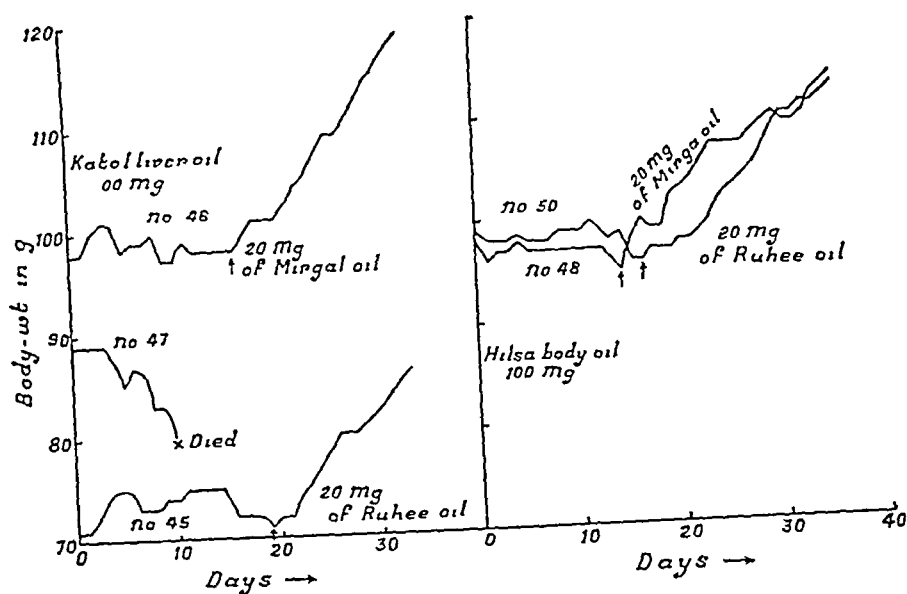


FIG 1

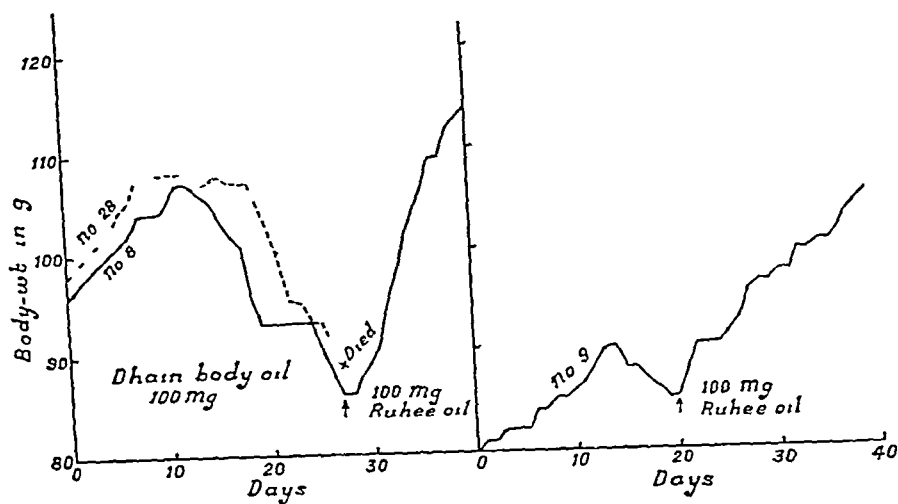


FIG 2

Fresh-Water Fish Oils.

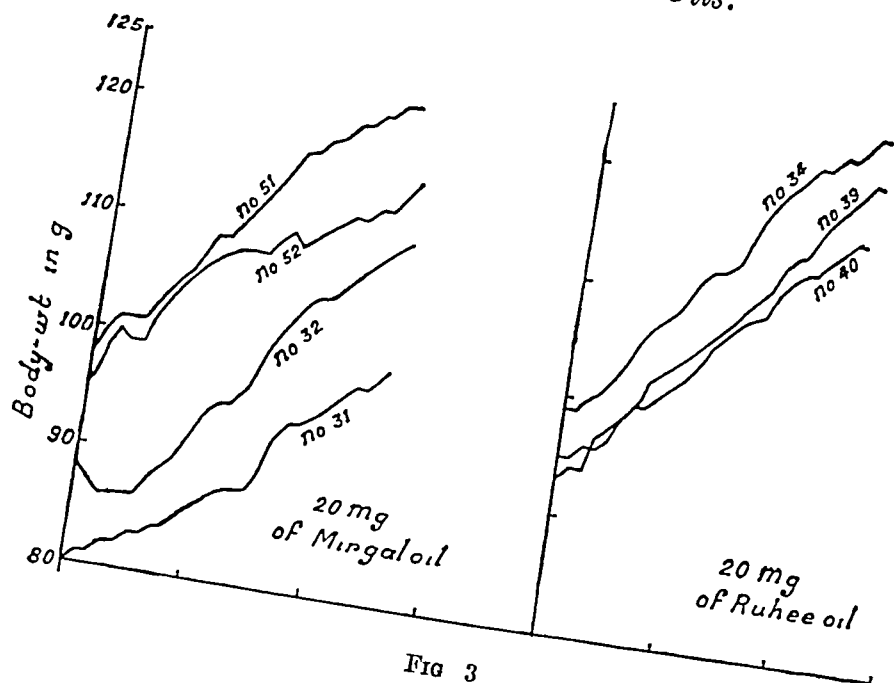


FIG 3

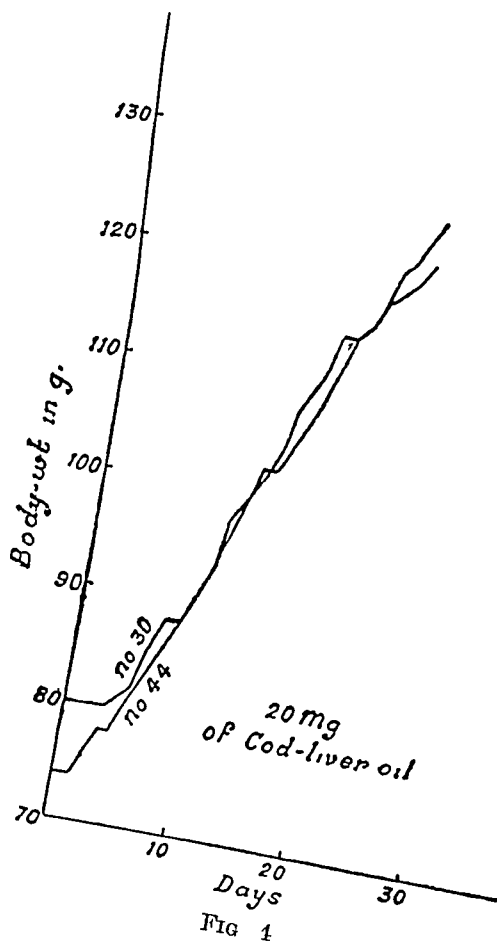


FIG 4

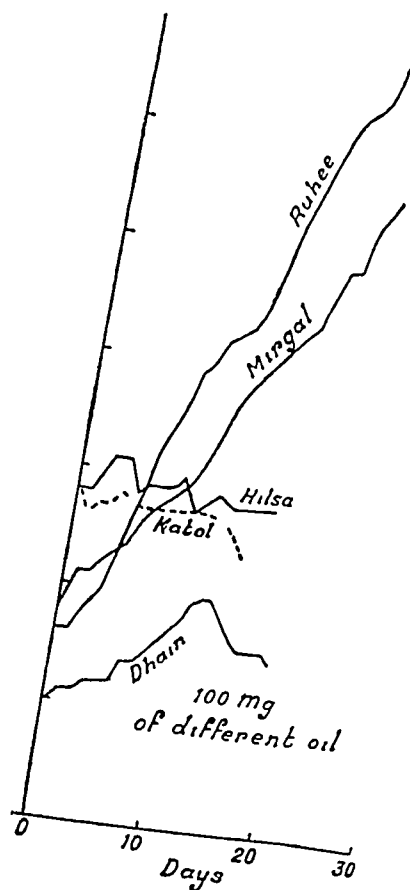


FIG 5

The dham-body oil did not produce any appreciable growth in doses of 100 mg. Four rats out of ten employed showed slight increase, others had a tendency to increase during the first two weeks followed by a rapid decrease. In the case of rats No 8 and No 9 (Fig 2) the supplement was changed to 100 mg of ruhee-body oil and the response was immediate. Rat No 28, without change of supplement, became blind and died on the 27th day (Fig 2).

The body oils of ruhee and mirgal were found to be potent in doses of 20 mg. The animals receiving these oils developed a healthy appearance after 2 weeks with perfect milk-white glossy fur (Fig 3).

The preparation of cod-liver oil used in this experiment induced a growth of 11.9 g per week, like average cod-liver oils (Fig 4).

Fig 5 shows the growth curve of rats on different oils in doses of 100 mg.

TABLE I

Showing the growth response of rats on different doses of oils

Nature of the oil	Number of rats used	Dose (mg)	Days under experiment	Average increase in weight per rat (g)	Average increase per rat per week (g)
1 Cod liver oil	5	20	28	47.7	11.9
2 Ruhee body oil	6	100	28	46	11.5
	4	50	28	30	7.5
	3	20	28	25.2	6.3
	4	20	35	30	6.0
	5	100	35	50	10.0
3 Mirgal body oil	4	50	28	30	7.5
	6	20	28	21.2	5.4
4 Dham body oil	10	100	14	5	2.5
5 Hilsa body oil	6	100	14	-8	
6 Katol liver oil	6	100	14	-10	

TABLE II

Showing the growth response of individual rats with 20 mg of different oils

Rat number	Sex	Days under experiment	Original weight (g)	Final weight (g)	Total increase in weight (g)	Total increase in weight per week (g)
<i>Cod liver oil</i>						
30	♂	28	80	128	48	12.0
41	♀	28	81	128	47	11.7
42	♂	28	73	123	50	12.5
44	♂	28	74	122	48	12.0
36	♀	28	95	140	45	11.2
					Average	11.9
<i>Ruhee-body oil</i>						
53	♂	21	97	115	18	6.0
54	♀	21	95	113	18	6.0
40	♀	28	93	116	23	5.7
39	♂	28	95	122	27	6.7
55	♂	21	71	89	18	6.0
35	♀	35	85	115	30	6.0
34	♂	28	99	125	26	6.5
					Average	6.0
<i>Mirgal body oil</i>						
31	♂	28	80	100	20	5.0
32	♂	28	88	109	21	5.2
33	♂	28	98	119	21	5.2
38	♂	28	74	97	23	5.7
51	♀	28	98	122	24	6.0
52	♀	28	95	116	21	5.2
					Average	5.4

The quantities of different oils required to promote the rate of growth of 3 g per week was taken as 1 animal unit as suggested by Sherman and used in the official method of vitamin A assay published in the United System of Pharmacopœia

TABLE III

Showing the relative vitamin A value of different oils (biologically)

Name of oil	Units of vitamin A per gramme	Weight of oil (g) which contains 1 unit of vitamin A
Cod liver oil	195-200	0.00502
Ruhee body oil	98-100	0.0102
Mirgal „	90	0.011
Dhain „	8-10	0.110
Hilsa „	Nil	
Katol liver oil	Nil	

Colour test — Reagent — A solution of antimony trichloride in chloroform was prepared by dissolving 30 g of purified antimony trichloride in 100 c.c. of chloroform at room temperature. Before being used the solution was cooled down to a temperature of 2°C to 4°C with ice and water and allowed to settle until a clear solution was obtained, since it has been pointed out by Wokes and Willmott (1927a, 1927b) that at room temperature the colour changes so rapidly that it is difficult to get an accurate reading.

A series of dilutions of the oils were prepared in chloroform. The colour produced when 0.2 c.c. of each dilution was mixed with 2 c.c. of the reagent was matched with standard Lovibond units of blue, the colours being matched after 30 seconds. The mean of three or more readings was taken in each case.

TABLE IV.

Showing colour produced when varying concentrations of different oils are treated with antimony trichloride

Nature of oil	Mg of oil in 0.2 c.c. of solution to 2 c.c. of reagent	COLOUR IN LOVIBOND		
		Blue	Yellow	Red
Cod liver oil (L. de Jongh)	5	1.0	0.2	
	10	2.1	0.6	0.1
	20	4.0	1.0	0.3
	40	7.5	2.3	1.0
	80	10.1	3.4	2.0
	100	11.3	5.0	4.0
Ruhee body oil	10	0.8	0.2	
	20	1.6	0.5	0.1
	40	3.0	1.0	0.1
	80	5.4	3.0	0.8
	100	5.8	3.2	0.9
	200	7.4	5.0	2.0
Mugal-body oil	10	0.7	0.2	
	20	1.4	0.5	
	40	2.6	1.0	0.1
	80	5.0	3.0	0.7
	100	5.4	3.4	0.8
	200	7.2	5.0	2.0
Dhain body oil	40	0.4	0.3	
	80	0.8	0.6	0.1
	200	1.6	1.1	0.3
Hilsa body oil	200	Nil		
Katol liver oil	200	Nil		

When the intensity of the colour produced is plotted against mg of oil used in the reaction a curve is obtained as in Fig 6 (a). In no case was the colour proportional to the amount of oil used. The curves were, however, found to approach a straight line at low values. The colour value equivalent to 1 animal unit of the cod-liver oil used in this experiment (0.00502 g), when used in the colour determination

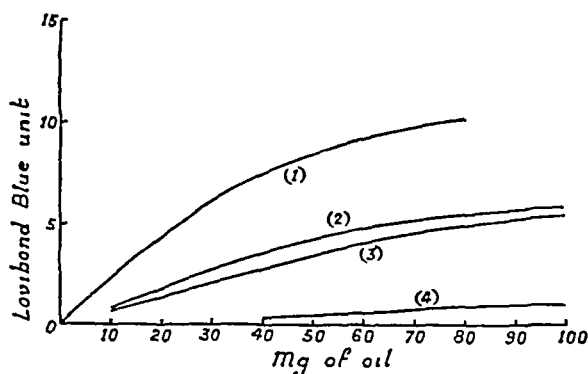


FIG 6 (a)

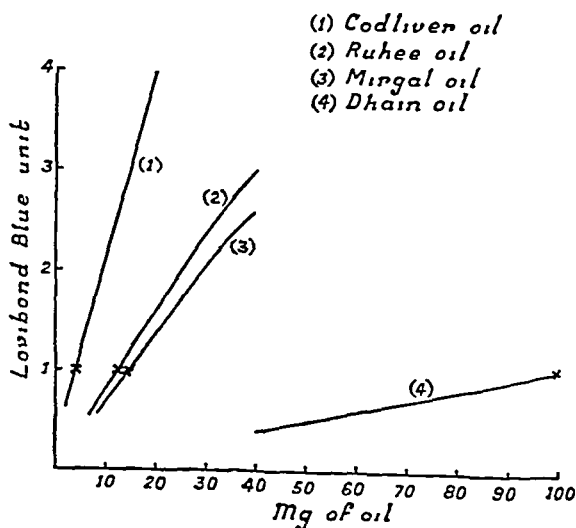


FIG 6 (b)

was found to give 1 Lovibond blue unit. The amount of each oil necessary to give the same colour may be obtained by plotting the lower values on a larger scale against mg of oil used for determination as in Fig 6 (b) so that a comparison can easily be made (Norris and Danielson, 1929).

TABLE V

Showing comparison of the amount of oil required to give 1 animal unit as determined by biological and colorimetric assay

Nature of oil	Biological assay (g) per day	Colorimetric assay (g) per day
Cod-liver oil	0 00502	0 005 (1 blue unit)
Ruheo body oil	0 0102	0 012
Mirgal body oil	0 011	0 014
Dhain-body oil	0 110	0 100
Katol liver oil		
Hilsa-body oil		

SUMMARY

1 The vitamin A potency of the different fresh-water fish oils as obtained by the biological method is in reasonably good agreement with those obtained by the colorimetric method

2 The body oil of ruheo and mirgal was found to contain 90 to 100 units of vitamin A. It is expected that the liver oils from the same sources will be much more potent in vitamin A. These fish which are extensively taken in food should form a very good source of vitamin A.

Our thanks are due to Professor V. Subrahmanyam, D.Sc., F.I.C., for the keen interest he has taken during the course of this investigation and also to Mr. K. D. Guha for supplying some samples of fish oils.

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ON SOME EXPERIMENTAL STUDIES ON LEPROSY

BY

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IN spite of the enormous effort put forth by workers all the world over in the experimental study of leprosy, it may be safely stated that, as yet, there is no clear evidence of any one having obtained successfully a culture followed by serial subcultures of the *Bacillus lepræ* (Hansen) associated with this disease in man. The positive cultures reported by some workers from time to time invariably lead to disappointment when attempts are made by others to confirm the findings with all due regard to the technical details prescribed.

Even from findings of experiments on animals, which have given somewhat hopeful results in the hands of some workers, the main evidence of bacillary proliferation is based on the recovery of the acid-fast microbes in the tissues and organs of certain animals (very often in abundance) after a varying period of inoculation of the human bacilli into them. This fact is open to the criticism, however, that the bacilli so recovered may be no more than the bacilli introduced from the human lesions, where they are so abundant, being somehow only gathered together in masses and then encapsuled, or that they may have been arrested while draining through lymphatic channels, and accumulated in certain situations like innocuous foreign body particles and that therefore they may not be the result of proliferation *in vivo* in the course of their pathogenic career, such as is evidenced in the case, for instance, of tuberculosis where bacillary proliferation is intimately associated with the specific cell reaction resulting in the production of a tubercle from which

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the bacilli are recoverable in culture. Such a criticism gains support from the findings of some of the workers (de Souza-Araujo, 1931) who have been able to demonstrate the presence of acid-fast bacilli in the tissues and glands of the animals into which they had introduced *B. lepræ* killed by heat (a finding we have confirmed in mice), or even an emulsion of killed acid-fast non-pathogenic microbes, like *B. phlei* (Fraser and Fletcher, 1914). There is, however, no evidence of this recovery of killed acid-fast bacilli being associated with any definite cell reaction or cell accumulation characteristic of any attempt at pathogenesis.

It is important to note on the other hand (leaving aside the results of inoculation with the so-called successful cultures of *Bacillus lepræ*), that many workers have reported on the production of nodules in different animals treated with human leprosy material, amongst them the following are prominent: Nicolle (1906), Duval (1911), Bayou (1912), Reensterna (1926), Franchini (1930), Schöbl *et al.* (1930), and de Souza-Araujo (1931). The value of the lesions reported by these investigators is lost to a certain extent by the fact that similar lesions have also been produced by them and others (Kedrowsky, 1918) by the inoculation of cultures of a variety of micro-organisms isolated from human lepromata, claimed to be cultures of *B. lepræ*, or of bacilli associated, at all events, with this micro-organism. Recently, Soule and McKinley (1932) have published a most interesting account of their success not only in obtaining serial cultures for eight generations (though tending to dwindle each time), but also of their having been able to produce by intracutaneous inoculation definite granulomatous nodules of identical structure in the macacus and ciba monkeys both with the human leprosy material and with their own cultures. The latest interesting contribution on the subject is that of Cantacuzene and Longhin (1932) who have been able to obtain acid-fast bacillary invasion of even extraperitoneal lymph glands in rats by intraperitoneal injection of filtrates of lepromatous crushings in saline, suggesting a filtrable virus responsible for this disease.

In the face of these historical published data the authors venture to submit the results of their experimental work which was primarily undertaken with the object of finding a stage in the experimentally-produced lesion which would be more favourable for obtaining definite cultures *in vitro*. Many of these results go to confirm the findings of some of the previous workers, but a few, being distinct and based on different methods of infection, are reported here with the hope of their throwing some further light on the obscure problem of this disease.

Although the intracutaneous method of infection has been adopted by Soule and McKinley (1932) and others as being more successful in inducing nodules than the intraperitoneal, intramuscular or subcutaneous methods, the present writers have confined themselves only to the subcutaneous and intraperitoneal methods of infection.

By adopting these routine methods, however, it was found impossible to obtain evidence of experimental transmission in rats, guinea-pigs and monkeys and sometimes even in mice, with human leprosy material, even after allowing several months for the disease to develop—a finding in conformity with that of Soule and McKinley (*loc. cit.*) who had also failed by the ordinary methods before obtaining successful lesions by intracutaneous methods instead.

It was therefore decided to alter the procedure of inoculation with the hope of rendering the animal more susceptible to infection. The plan consists of an intensive method of infection by giving repeatedly weekly injections by the peritoneal route alone (in the case of mice, rats and guinea-pigs) and then following these up (in the case of monkeys) with subcutaneous injections, and it may be stated here at once that this has been fruitful (at least in mice and monkeys) in inducing a condition more favourable for the disease germ to implant itself into the tissues or to induce at least a local reaction. The following is a brief account of our experiments —

The standard fluid used is a citrated saline suspension collected in a test-tube from the crushings of a freshly excised human lepromatous nodule (about 1 cm in diameter and 0.5 cm in thickness in 20 c.c. of the fluid). It is uniformly opalescent and is rich in *B. lepræ* in bundles and individuals, and free from the large masses of bacilli which sink to the bottom of the tube, agglutinated and entangled in a loose coagulum.

EXPERIMENTAL OBSERVATIONS

White mice — These animals bear comfortably a dose of 0.25 c.c. of the infecting material (suspension) intraperitoneally. They remain practically normal in habits and appearance, although in some instances one finds intercurrent abscesses, lung tumours, even bronchopneumonia (6 in one of our series of 26)* unconnected with the inoculation. Of those free from such complications (20 out of 26)* though showing no external signs of any disease, 16 revealed on opening the abdomen one or two minute nodules—no larger than half of a fig seed, greyish white in colour, well defined and situated in the omentum near the spleen or in the gastrohepatic omentum, or even in the mesentery. (Those observed after a year were white in colour and more numerous.) They were easily removed from their bed, being firm in consistency. These seeds make their appearance even a month after the last injection in an animal prepared by six to eight weekly intraperitoneal injections and (from one of the experiments recorded) appear to grow in number though not in size by age. They also seem to become more prominent after some months, being white and hard owing to calcium deposit. When punctured in a drop of normal saline solution such a seed or nodule yields an opalescent fluid which when smeared and stained shows a richness of fat and apparently vigorous bacilli as though smeared from a bacterial culture tube (Plate XXV, fig. 1). These when planted on various media, however, fail to yield cultures.

A section of a nodule shows that it is made up of a conglomerate of microscopic, more or less spherical, units separated from the neighbours by loose fibrous tissue, the whole encapsuled in a similar structure and containing lepromatous cells, connective tissue-cells and abundance of *B. lepræ*. The body of the nodule is a spongework of leproma cells and bacilli which have apparently proliferated and after a time have been packed in the interstices (Plate XXV, figs 2, 3 and 4).

* See Appendix

The disposition of the connective tissue-cells (most of which are lepromatous) in the loose spongework and in between the septa somewhat concentrically arranged, and the abundance of *B lepræ* packed in the nodules leads one to suspect that there has been a regular proliferation of the bacilli and that the massive accumulation of the cells of a particular nature and structure indicated the formation of a granuloma characteristic of the disease, and that the whole lesion is well encapsuled in an effort to isolate it from the surrounding tissues.

In almost all cases where the lesions are recent only one seed-like body is found, but in one animal which had survived over 15 months, 5 or 6 seeds were detected and were picked up easily, they were chalky white in appearance—a condition ascertained as being produced by calcium deposit in the spongework.

This observation supports the suspicion of the proliferation of *B lepræ* and definite cell accumulation which becomes more pronounced in animals surviving for a long time. The peritoneum throughout shows no evidence of acute inflammation or adhesions. The lymphatic glands in the upper part of the mesentery are sometimes found slightly enlarged, especially in those cases where the seeds are not detected, but in all cases the crushings from the glands when smeared on slides and stained reveal acid-fast bacilli (although very sparingly distributed) in the midst of lymphocytes and mononuclear cells, some of which contain occasionally 5 to 6 acid-fast bacilli. In some sections of these glands cell collections are demonstrable as definite granulomata, and in others lymph-spaces, in which are found masses of globi (Plate XXVI, figs 5, 6, 7 and 8).

The spleen is always enlarged but free from acid-fast bacilli, and sections show no definite granulomatous collections of cells as seen in the lymphatic glands. In all cases, however, large giant cells are prominent. These findings in white mice go to confirm some of the results of de Souza-Araujo, although the lesions we have obtained are constant in them being small in size, their being found at definite seats of election, and their specificity in structure.

White rats—Injections given intraperitoneally with a weekly dose of 1 cc for over 10 weeks have failed to induce any definite lesions even after an interval of two months of the last injection. The only evidence of the inoculation is the presence of a few scattered bacilli (acid-fast) in the smears made from the mesenteric glands which when examined in sections show lymphocytic hyperplasia with a large number of giant cells in all stages but without any definite granulomatous formation. The sparing number of bacilli may be only the dead ones arrested and unaltered in the lymphatic glands, and acting only as mechanical particles (Plate XXVII, figs 9 and 10).

Guinea-pigs—These animals treated similarly have yielded nothing of importance in all the experiments. They behave exactly as the white rats, with a sparing number of acid-fast bacilli in the mesenteric glands which show no alteration of structure beyond a slight increase in size.

Monkeys—*Macacus rhesus* used in all the experiments is found refractory to subcutaneous or intraperitoneal injections as such, of lepromatous crushings in



Fig 1 Smear of saline crushings of an omental nodule, mouse 28 Ziehl Neelsen, $1/12 \times 10$ ocular



Fig 2 Section of an omental nodule to show cellular structure and lymph spaces Hæmatoxylin, C $\times 10$ ocular



Fig 3 Section of omental nodule, mouse 25, to show the general arrangement of fibrous strands and cells and acid fast bacilli Ziehl-Neelsen, C $\times 5$ ocular



Fig 4 Part of same as in Fig 3 Ziehl Neelsen, $1/12 \times 5$ ocular



Fig 5 Section of mesenteric gland, mouse 28, to show early and late granulomatous formation (lighter parts) Hematoxylin, C $\times 5$ ocular

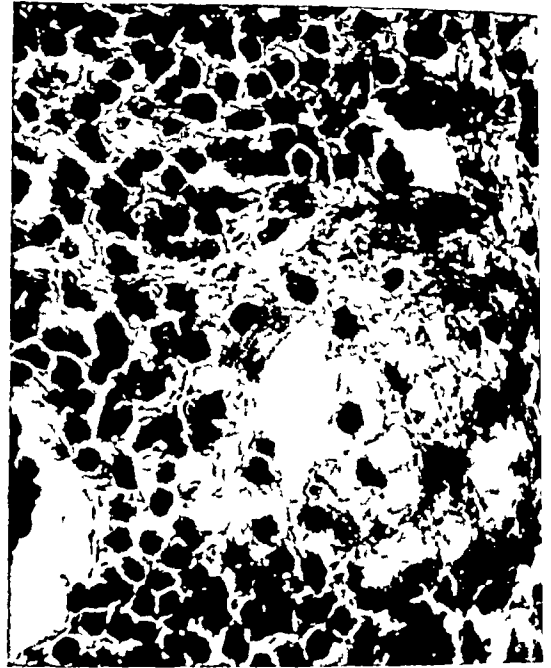


Fig 6 Part of same as in Fig 5 Hematoxylin, $1/12 \times 5$ ocular



Fig 7 Section of mesenteric lymph gland, mouse 26, to show the general lymphoid hyperplasia and lymph-spaces filled with globi Ziehl-Neelsen, C $\times 5$ ocular

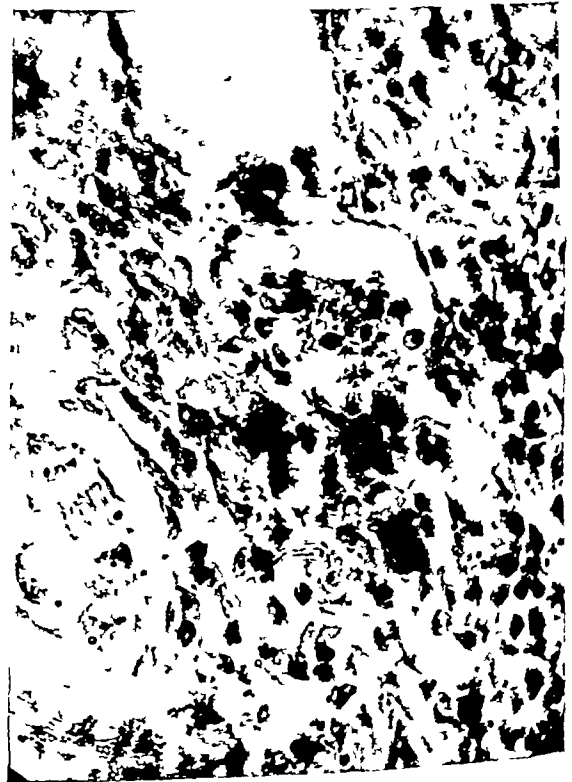


Fig 8 Part of same as in Fig 7 Ziehl-Neelsen, $1/12 \times 5$ ocular

PLATE XXVII

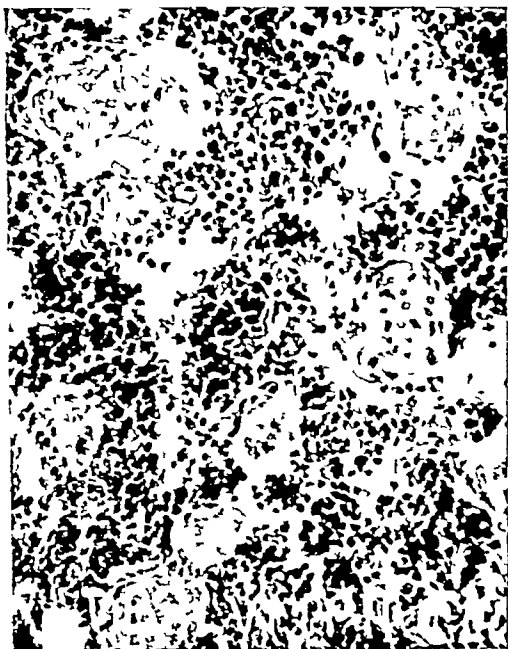


Fig 9 Section of mesenteric lymph gland, rat 'B', to show lymphoid hyperplasia and giant cells, C $\times 10$ ocular

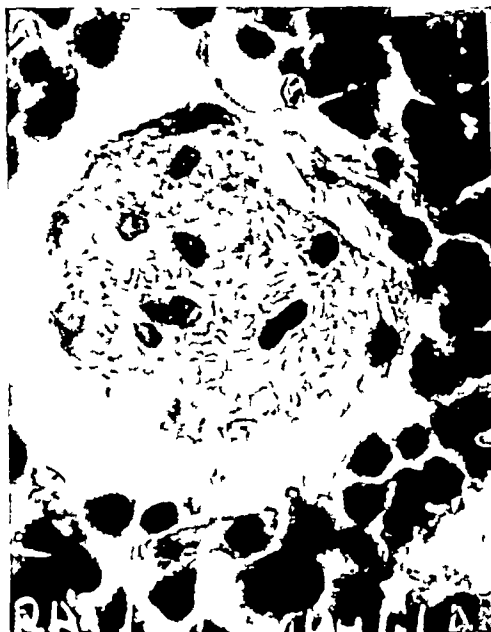


Fig 10 Part of same as in Fig 9 to show a giant cell, 1/12 $\times 10$ ocular



Fig 11 Lepromatous nodules in the forehead of *Macacus rhesus* (L 2), 3 weeks old



Fig 12 Lepromatous tumour in the abdominal wall (L 3) *Macacus rhesus*, 3 weeks old

(2) Definite pathological lesions are produced by an intensive method of infection, which consists of several intraperitoneal injections repeated week after week, but these effects are not the same for all the animals

(3) Guinea-pigs and white rats remain refractory to intraperitoneal injections and show no obvious lesions. Their mesenteric glands, however, become slightly enlarged and reveal lymphocytic hyperplasia rich in giant cells with only a very sparing distribution of acid-fast bacilli but no definite granuloma formation

(4) White mice develop after 6 weeks or more even with a course of 4 to 6 intraperitoneal injections, definite, though minute, seed-like nodules situated as a rule in the gastrosplenic omentum, sometimes in the gastrohepatic omentum, and occasionally in the mesentery. These nodules are made up of aggregations of cells enclosed in a fibrous capsule, and packed with apparently vigorous acid-fast bacilli, suggesting a well-marked bacillary proliferation. Mesenteric glands show definite specific granulomata, but with less pronounced bacillary invasion

Boiled human lepromatous material similarly injected 6 to 8 times intraperitoneally into mice fails to produce nodules but acid-fast bacilli, sparingly distributed are demonstrable in the mesenteric lymph glands which show distinct lymphocytic hyperplasia

(5) Macacus monkeys remain apparently well after a course of several intraperitoneal injections. But after a course of 8 to 12 such injections repeated weekly they become sensitive to subcutaneous introduction of human leprosy material which each time excites in them (after 15 to 20 days) a remarkable specific granulomatous tumour formation (progressing for several weeks before their gradual but complete absorption) with characteristic structure, but with only scanty distribution of acid-fast bacilli—in contrast with the lesions in mice where the ratio of cell proliferation to the bacillary content is reversed

(6) Cultures *in vitro* from human as well as from experimental lesions at different stages of their development have so far entirely failed

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APPENDIX

Mouse number	Post mortem date	Number of injections	Period of injections	Lesions	Results and findings
20†	23-1-33	3	15 months ago 30-10-31 to 13-11-31	Multiple nodules	Abundance of <i>B lepræ</i> in nodules
24*	23-2-33	10	30-11-32 to 18-1-33	Nodules, 2	Do
25†	24-2-33	10	30-11-32 to 18-1-33	Nodules, 3	Do
					Sections show <i>B lepræ</i> masses Lymph spaces enlarged
27†	8-3-33	10	30-11-32 to	{ No nodule	Negative
28†	28-3-33	10	18-1-33	{ Nodules	Full of <i>B lepræ</i> Glands granulo matous Sections also full
32*	25-3-33	6	1-2 to 22-3-33	Nodules	Do
33†	13-4-33	6	1-2 to 22-3-33	No nodule	Negative
34†	18-4-33	6	1-2 to 22-3-33	Nodule, 1	Lymphatic gland en- larged, full of <i>B lepræ</i>
35†	19-4-33	6	1-2 to 22-3-33	Nodules, 2	Do
45†	28-4-33	4	30-3 to 19-4-33	No nodule	Abdominal abscess shows <i>B lepræ</i> and streptococci
46†	1-5-33	4	30-3 to 19-4-33	Nodule, 1	Full of <i>B lepræ</i>
47*	14-5-33 decomposed	4	30-3 to 19-4-33	Nodule, 1	Full of <i>B lepræ</i> , septic Scrotal abscess Inguinal gland <i>B lepræ</i>
48†	17-5-33	4	30-3 to 19-4-33	No nodule	Mesenteric abscess Pus shows <i>B lepræ</i>
49*	16-5-33	4	30-3 to 19-4-33	Decomposed	
50†	24-5-33	6	30-3 to 10-5-33	No nodule	Negative
51†	29-5-33	6	30-3 to 10-5-33	Tiny nodule	Full of <i>B lepræ</i>
52†	29-5-33	6	30-3 to 10-5-33	No nodule	Patch on liver full of <i>B lepræ</i>

*Died

†Killed

APPENDIX—concl'd

Mouse number.	Post-mortem date	Number of injections	Period of injections	Lesions	Results and findings
54†	7-6-33	6	30-3 to 10-5-33	No nodule	Full of <i>B lepræ</i>
55†	8-6-33	6	30-3 to 10-5-33	Nodule and gland	
56†	22-6-33	6	30-3 to 10-5-33	Nodule	
63†	1-6-33	5	26-4 to 31-5-33	Nodule	
64*	12-6-33	6	26-4 to 7-6-33	Nodule	
65†	12-6-33	6	26-4 to 7-6-33	Nodule	Do
66	Died of an accident		No post-mortem		
67†	12-6-33	6	26-4 to 7-6-33	Nodule	Full of <i>B lepræ</i>
68†	7-6-33	5	26-4 to 31-5-33	Nodule	Do

*Died †Killed

THE EFFECT OF A PLASMODIAL INFECTION IN INCREASING SUSCEPTIBILITY TO LEISHMANIA INFECTION IN MONKEYS

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CLINICAL and epidemiological observations have led to the enunciation of the hypothesis that kala-azar in man is always secondary to some infection which determines his susceptibility and that in India this infection is usually malaria or typhoid, this hypothesis has been supported by a number of laboratory observations which we, the present writers and their co-workers, have reported from time to time

In certain preliminary experiments it was noted that *Macaca irus* monkeys that had been infected with a monkey plasmodium were very susceptible to leishmania infection, and it was decided that a series of experiments with controls should be carried out to ascertain if there was any evidence that the malarial infection increased the susceptibility of these animals

Twenty-four monkeys were employed in this series of experiments, the number employed was limited not so much on account of the expense of obtaining the monkeys as on account of the difficulty of caging and housing a larger number of animals for the comparatively long time that it is necessary to keep these animals under observation

Two species of monkey were used, *Macaca mulatta* and *Macaca irus*. The results of the experiments with each species will be discussed separately

Macaca mulatta

These were divided into three groups each consisting of three monkeys. Group A monkeys were inoculated with the plasmodium, but this infection was kept in check by the giving of quinine (or atebirin) for about three months, two were then inoculated intraperitoneally with doses of 0.5 and 1.0 c.c. of a spleen and liver emulsion of a leishmania-infected hamster and one monkey was inoculated subcutaneously with 1.0 c.c. of the same emulsion. Group B monkeys were similarly inoculated with leishmania, but without any previous plasmodium inoculation. Group C monkeys were first inoculated as above with the leishmania-infected material and then two months subsequently with the plasmodium infection, one of the monkeys in the last group died of malaria despite treatment.

Results—Two other monkeys died at two and three months respectively after the leishmania inoculation, and the others were sacrificed after about six months, smears and cultures being made from their organs. There was no evidence of leishmania infection in any of these monkeys.

Macaca mus

The monkeys were similarly divided into three groups, the difference being that there were five monkeys in each group, that three in each group were given intraperitoneal injections and two subcutaneous injections, and that in group A cases the leishmania was given 10 days after the plasmodium inoculation in three and one month and six weeks, respectively, in the other two.

Result—Group A One monkey died within six days of the leishmania inoculation and should not be considered. Three out of the remaining four became infected with leishmania which infection was demonstrated after 3, 4 and 8 months, respectively, and the fourth was found dead seven months later, he had an enlarged spleen, but no leishmania could be found in the smear.

Group B Three of these were found infected at the end of 3, 7 and 11 months, respectively, two were sacrificed after 9 and 12 months, respectively, and were found negative to both smear and culture.

Group C One was found infected after 8 months and one that was found dead after 5 months was negative to smear examination, three were sacrificed after 8 and 9 months, respectively, and proved negative culturally.

These results are very inconclusive. In the *M. mulatta* groups there is no evidence of any increased susceptibility following malarial infection. In the *M. mus* groups, comparing group A with the other two groups we see that there were in groups B and C five monkeys which were proved to be uninfected as against none in group A (as in the case of the monkey in this group that died no culture was possible). Thus, taking the most optimistic view and leaving out of account the monkeys that were not examined culturally, we have three out of three positives in group A, i.e., following plasmodium infection, and four out of nine positives in the other groups, but it is doubtful if even this difference can be considered significant as the numbers are so small. As the 'control' monkeys show some susceptibility to leishmania infection, the experiment would have to be repeated on a very large scale before one could hope to show any difference in susceptibility that could be considered significant.

HÆMATOLOGICALLY CONTROLLED EXPERIMENTS

As *M. vrus* monkeys are frequently found infected with this plasmodium in nature and as they show little morbid reaction to infection it might be argued that they have all had malaria at some time or other and are consequently all susceptible to leishmania. We therefore decided to attack the problem from a slightly different point of view.

It has been shown in a previous paper (Krishnan, Lal and Napier, 1933) that there is a considerable difference in the histiocyte response to plasmodial infection on the part of different individual monkeys, and we have suggested that it is with this histiocyte response that the susceptibility to leishmania infection is associated. We therefore decided to select monkeys for these experiments in which there was a marked histiocyte response and choose the time for inoculation when this response was at its height.

Four such monkeys (*M. vrus*) were chosen at times when their histiocyte counts were between 15 and 25 per cent, two were given 0.5 c.c. of spleen emulsion from a leishmania-infected hamster, and two were given 0.1 c.c. of a flagellate culture of leishmania.

Result—All four became infected, in three the infection was demonstrated after four months and in the other the liver puncture was 'doubtful' at four months but positive at 8 months.

Controls—There were unfortunately only two controls in this experiment, one was a monkey which had been infected with malaria, but in which there was no histiocyte response, and the other was a normal monkey, they received the same doses of spleen emulsion and flagellate culture, respectively. At eight months they were sacrificed and both smears and cultures were negative.

In this second experiment the results were more clear-cut, but again the numbers were small.

CONCLUSION

M. mulatta monkeys enjoy a degree of immunity to infection with *Leishmania donovani*, by previous or subsequent inoculation with a plasmodium infection, which was kept in check by quinine administration, we were not able to reduce this immunity to any appreciable extent.

M. vrus monkeys possess a lower degree of immunity to leishmania infection, and there is some—though not very conclusive as the number involved in the experiment was small—evidence that this is lowered during the process of response to a plasmodial infection.

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DERMAL LEISHMANIASIS IN ASSAM

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[Received for publication, July 26, 1933]

THE first case of post-kala-azar dermal leishmaniasis was reported in 1922, during the next few years single cases were reported, all of them from Bengal. In 1927 a series of 44 cases, seen during the previous year at the Calcutta School of Tropical Medicine, was reported, and a second series of 150 cases was reported from the same institution in 1930, again these were all from Bengal. In 1928 a case was reported from Assam and a second one in 1929. A few other cases had been seen in Assam, but in the present writers' combined experience in the province, which included a number of years of the epidemic period in a heavily kala-azar-infected area, some four or five cases only were seen by them. Dey (1929), who reported the second case referred to above, wrote, 'In my experience extending over a period of four years on kala-azar duty in the most heavily-infected areas of Nowgong (Assam) I have come across only this one case.'

As during the last 10 years the total incidence of kala-azar in Assam has been much greater than that in Bengal, relative to the population, this comparative rarity of the disease in Assam seemed to require some explanation. Both the writers had had personal experience in the province, but nevertheless they had never undertaken any systematical search for cases of this condition. In Madras from where few cases had hitherto been reported but where the relative incidence of kala-azar was much less, a number of cases of dermal leishmaniasis were found when the matter was especially investigated

We therefore decided to visit Assam with the object of finding out whether this rarity of dermal leishmaniasis was real or apparent. It was arranged that the senior writer (L E N) should visit tea-gardens and examine the labour forces, and that the junior writer (R O A S) should tour the villages in the endemic area, paying special attention to places where there had been, or still were, kala-azar treatment dispensaries.

VILLAGE INVESTIGATION

The junior writer (R O A S) visited the villages around Gauhati and Goalpara that were reported as having been very heavily infected during the epidemic years. He made inquiries from the local doctors, from the kala-azar and leprosy dispensaries, and from the large hospitals, he visited 'hâts' where in a few hours he was able to see thousands of people, and in a few instances visited the villages themselves where he saw as many people as he could persuade to come out of their houses, in some of these villages he was told that the whole population had received treatment for kala-azar. Altogether he must have seen over 5,000 people sufficiently closely to spot any facial lesions, and some 500 which he was able to examine more closely.

In one village of between 300 and 400 inhabitants 310 persons had been treated for kala-azar, in this village 80 persons who had had kala-azar were examined and one case of dermal leishmaniasis found. Again at a large 'hât' in the centre of an intensely-infected area where the local dispensary had treated 4,999 since 1919, one case was spotted out of not less than 1,500 persons.

Altogether the junior writer found three cases himself and had his attention drawn to three others by local medical men. He also heard of a few other cases in which this diagnosis had been made, but he was not able to see them and confirm the diagnosis.

Of the six cases seen four were indigenous inhabitants of Assam, two were of Bengali stock but had lived in Assam for many years.

TEA-GARDEN INVESTIGATION

We had previously circularized the doctors in districts in Assam where kala-azar was known to be prevalent and had obtained promises of co-operation. Here therefore it was possible to make a closer personal search for individuals suffering from dermal leishmaniasis. The procedure that was adopted was to line up as many coolies as possible, and to make the men and children uncover their bodies down to the waists, the senior writer then passed slowly down the line examining the faces, hands and arms, abdomens, chests and backs, and thighs, legs and feet of each individual; the women were told to loosen their clothes so that their backs could be examined easily and they were then examined in the same way except that the examination of their chests was limited to parts above the breasts. The manager of the garden and the assistant medical officer were present and pointed out any persons who were known to have had kala-azar, a count was kept of the number examined and a separate count of those known to have had kala-azar.

Results of the investigation on seven gardens where this procedure was adopted are given in detail in the following Table —

TABLE
Dermal leishmaniasis in Assam

Tea garden	Population (about)	Coolies seen at muster	Coolies pointed out as having had kala azar	Number of cases of dermal leishmaniasis	Kala azar incidence	Since	REMARKS
A	1,400	800	218	0	487	1922	Steady incidence, last year 74 cases
B	900	Many	9	0	High	1922	
C	2,000	1,207	145	1	393	1926	Heavily infected from 1919
D	1 100	609	50	0	267	1923	
E	1 500	496	35	0	105	1927	Previously very heavily infected
F	2,500	70	70	0	400	1923	
G	1,000	503	40	0	High	1924	

Most of these gardens were in the Nowgong area. The definite case of dermal leishmaniasis (confirmed microscopically) that was found was a Mikir (an aboriginal from the Mikir Hills, Assam), who had worked on the garden for a number of years. He was not amongst the coolies pointed out as having had kala-azar, but when questioned he admitted that he had suffered from a fever for which he had received a number of injections at a dispensary in the neighbourhood.

We did not take into account the place of origin of the coolies examined, short-term coolies and those that had only recently arrived were not usually examined and are at any rate not included in the figures given in the Table. Many were indigenous inhabitants of Assam but the majority were of immigrant stock though they had been born, or had been living for some years on the gardens. There is very little, if any, recruitment of labour for the tea-gardens in known kala-azar endemic areas.

In other gardens where it was not convenient to examine a large number of coolies in this way, a few that were known to have had kala-azar were collected at the hospital and examined. In all the gardens visited the assistant medical officers were questioned carefully as to the occurrence of the lesions of dermal leishmaniasis and in many instances patients with various skin lesions were produced for examination.

In the Golaghat area amongst the gardens visited were two where during the epidemic years there had been a very high incidence of kala-azar, in one garden there had been 350 cases in a total population of about 1,700 during the first few years of the epidemic period, and in the other, a garden with a population of about 2,800, there had been 367 cases between 1921 and 1924. The medical officer, a well-qualified Indian doctor, had been employed especially on these two gardens to deal with the kala-azar cases throughout the epidemic period and was now in medical charge of the gardens. He had been on the lookout for the dermal lesions and had diagnosed four such cases. He was able to produce three of these patients, and there was no possible doubt about the correctness of the diagnosis. In the fourth case the condition had apparently cleared up. A particularly interesting point about these patients was that three were related to one another and that they were all imported from the same district in Orissa, namely, Sambalpur—a place which, though not generally recognized as a kala-azar endemic area, is not far from the heavily-infected endemic areas in Bengal. These patients contracted kala-azar on the garden but very soon after their arrival, and the medical officer stated that many coolies had arrived from this district actually with the infection. A similar statement was made by other medical officers regarding Sambalpur coolies.

A number of gardens were visited in the Bokakhat, Mariani and Tinsukia areas, in these areas the disease has never assumed epidemic proportions and in the last-named particularly the cases of kala-azar seen or reported were mostly imported. No cases of dermal leishmaniasis were seen or reported in these areas.

A few 'hāts' were also visited, but again no cases were found.

DISCUSSION

Although it is not allowable to base any far-reaching conclusions on negative observations, it can, we feel, be claimed after a systematic examination of about 4,000 coolies of whom at least 567 were known to have had kala-azar, with the finding of only one case of dermal leishmaniasis, that the condition is rarer than it is in Bengal or Madras. Comparison can be made with the finding of 6 cases in 120 persons examined in Bengal (Napier, 1931), and 5 cases in a comparatively small village area in Madras where systematic examination was not carried out (Napier and Krishnan, 1933).

The other observations provide contributory evidence, but in addition they suggest that it is the western parts of the Assam valley that provide such cases of dermal leishmaniasis as are to be found in the province.

The observations provide further evidence for the theory that the dermal lesions are phenomena of host-parasite adjustment. There is considerable evidence to show that kala-azar first invaded the western end of the valley, here cases of

dermal leishmaniasis are to be found, though comparing the incidence with that in Bengal they are rare. In the Nowgong district where kala-azar is known to have existed at least 40 years one case was found, but there is little evidence of the existence of dermal leishmaniasis amongst the indigenous population further east, in the districts invaded by kala-azar in comparatively recent years, though admittedly our personal investigations were not so thorough. The few cases encountered were amongst coolies imported from a place which is geographically much nearer to the old endemic areas in Bengal (and which indirect evidence suggests is an endemic area itself).

Our thanks are due to the Inspector-General of Civil Hospitals, to the Director of Public Health, to many Civil Surgeons and Special Kala-azar Officers, and to a number of tea-garden Medical Officers and their assistants for information and valuable assistance in this inquiry.

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EFFECTS OF INSULIN ON THE CONTRACTIONS OF THE INTESTINAL MUSCLE

BY

S PRASAD, B SC, M B

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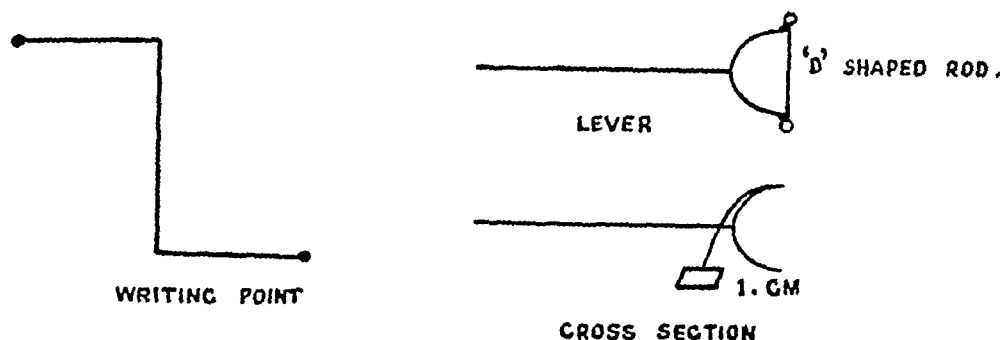
[Received for publication, August 7, 1933]

THE experiments were performed on the isolated intestinal muscle of full grown rabbits, fed on normal diet. The animals were killed by air embolism. A piece of small intestine, distal to the duodenum, was rapidly isolated from its mesenteric attachments, cut and immersed in oxygenated Tyrode solution. The Tyrode solution used was that employed by Magee and Reid (1927) as this was found most suitable for rabbits here. Instead of a Burn-and-Dale bath, a special bath of galvanized tin, paraffinized internally, was improvised locally. It consisted of a central chamber where the intestine was hung in oxygenated Tyrode solution. The central chamber was surrounded by a water-jacket to keep the temperature of the Tyrode solution constant at 38°C. The whole bath was placed on a wooden stand, which was fitted with electric bulbs for keeping the temperature constant.

In order to have a frontal writing point, a special writing point was improvised as shown in the Text-figure. To the lever made of a thin and light rectangular aluminium rod, a D-shaped thin brass-rod was attached. The vertical limb of the D-shaped rod was joined to the curved limb by hinges. To the centre of the vertical limb was attached the writing point made of fine glass capillary. It had a vertical and two horizontal limbs. The centre of the vertical limb was fixed to the vertical limb of the D-shaped rod. The end of the lower horizontal limb was ball-pointed and was made to write against a slow moving drum of an improvised kymograph (Narayana, 1931). To the upper horizontal limb was attached a weight of one gramme to keep the lower limb against the smoked surface. The above bath and writing

564 *Effects of Insulin on the Contractions of the Intestinal Muscle*

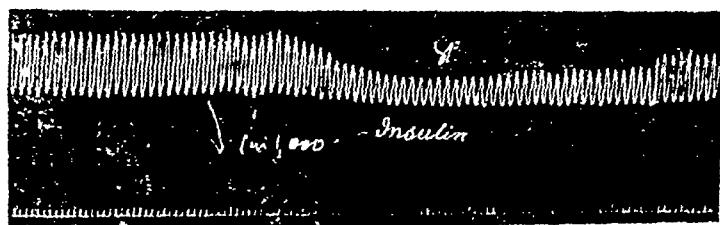
point have been used satisfactorily for a number of experiments where record of intestinal contraction was necessary (Prasad, 1933)



TEXT-FIGURE

After the isolated piece of intestine was hung in the oxygenated Tyrode solution as mentioned above, it was allowed to contract for about half an hour so as to ensure regular and systematic contractions before insulin was administered. All these contractions were recorded. The drug was then added in the quantity requisite to make up concentrations of 1 in 10,000, 1 in 5,000 or 1 in 1,000, etc., as required. It was found that weak dilutions such as 1 in 10,000 showed very little or practically no effect either on the amplitude of contraction or rate of movements or tone of the muscle. A dilution of 1 in 5,000 had some effect in diminishing the amplitude of movement but no effect on the tone of the muscle could be noticed. A dilution of 1 in 1,000 diminished the amplitude of movement and also the tone of the muscle to some extent (Curve 1)

CURVE 1

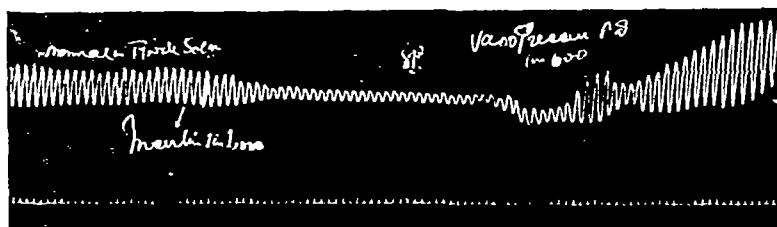


Insulin 1 in 1,000 added at the arrow mark
Time tracing at interval of 3 seconds

The diminished amplitude continued for about 5 minutes when recovery took place. Complete return to the original amplitude of movement and tone of the muscle was seen in 5 to 10 minutes depending upon the concentration in which insulin was added. The rate of movement was not affected.

The recovery process, after addition of insulin, was hastened by the addition of vasopressin, the tone of the intestinal muscle being raised and the amplitude of movement being increased to a marked extent (Curve 2)

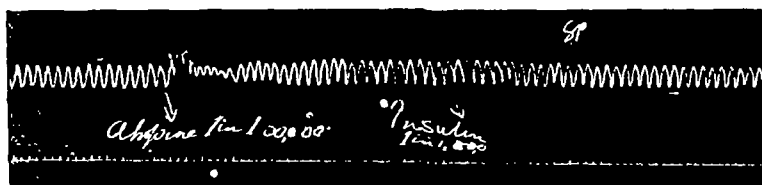
CURVE 2



Insulin 1 in 1,000, later vasopressin 1 in 600 added
Time tracing at interval of 3 seconds

The effect of insulin on the intestinal movements was abolished by previous addition of atropine in suitable concentration. After adding atropine to the fluid in concentration of 1 in 1,000,000 the subsequent addition of insulin 1 in 1,000 had little effect. But addition of atropine in concentration of 1 in 100,000 practically abolished the effect of insulin 1 in 1,000 (Curve 3). The action of insulin therefore appears to be on the nerve-cells or nerve-endings rather than directly on the muscle. Insulin appears to act as a depressor of the vagal system as the effect is abolished by the addition of atropine.

CURVE 3



Atropine 1 in 100,000, later insulin 1 in 1,000
Time tracing at interval of 3 seconds

Further, it was found that the utilization of glucose, present in the Tyrode solution used, by the isolated intestinal piece was decreased under insulin. Two series of experiments were carried on. In one series the intestine was allowed to contract for three hours in oxygenated Tyrode solution without any drug to serve as a control. In the other series insulin was added, usually in three doses, each time in a quantity sufficient to make up a concentration of 1 in 1,000 in the Tyrode solution. The intestine was allowed to recover and contract normally for some time

before the second dose of insulin was given. It was found that the amount of glucose utilized per 100 grammes of the intestinal piece in the first series, varied from 300 to 400 milligrams, whereas in the second series the amount of glucose utilized per 100 grammes of the intestinal piece was decreased, varying from 100 to 200 milligrams as shown in the Table below. This was due to the fact that the contraction of the intestine, in the second series, was interfered by the addition of insulin. The glucose in the solution was estimated by the method, described by Shaffer and Hartman (1920-21) for determining small amounts of sugar. In order to avoid the presence of insulin, mucus and other albuminous matter in the solution, filtrates were obtained by employing the Folin Wu tungstic acid precipitation.

TABLE
*Glucose utilization per 100 grammes
of intestine*

	Control, mg	Under insulin, mg
24-2-32	285	187 3 doses
26-2-32	346	205 3 „
28-2-32	400	230 3 „
2-3-32	397	212 2 „
8-3-32	299	153 3 „
4-3-32	397	212 3 „
14-3-32	410	102 4 „

SUMMARY OF RESULTS

Insulin in a concentration of 1 in 10,000 had no effect on rate or amplitude of contraction or tone of the muscle
 „ „ 1 in 5,000 caused diminution in amplitude
 „ „ 1 in 1,000 caused greater diminution in amplitude of contraction. Tone of the muscle also lessened.

These findings corroborate the finding of Winter and Smith (1923) who also noticed these effects of insulin.

CONCLUSION

Insulin in appropriate concentration lessens the amplitude of contraction of isolated intestinal muscle of rabbits. It also lessens the tone to some extent. Later on the normal amplitude and tone are regained. The recovery process is hastened by the addition of vasopressin. Insulin perhaps acts on the nerve-cells or nerve-endings as the effect is abolished by the previous addition of atropine.

The insulin used was the product of Eli Lilly & Co, U S A, containing 20 units per c c.

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OBSERVATIONS ON FILARIASIS IN SOME AREAS IN INDIA

Part X

GWALIOR STATE AREA

BY

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[Received for publication, August 16, 1933]

IN continuation of the inquiry into the distribution of filarial infection which has been carried out in different parts of India presenting different geographical and climatic conditions, an investigation into filariasis in areas of Gwalior State in the Central India Agency was undertaken under the auspices of the Indian Research Fund Association, between the dates of 26th January and 13th February, 1933

From the epidemiological viewpoint, it was considered important to observe the physical characters that favour the spread or restrict the progress of the disease, for, although the infected areas are shown to be widespread in India (Acton and Sundar Rao, 1931, *see* Map of India), heavy filarial incidence appears to be restricted to areas possessing certain definite physical conditions

While studying filariasis in the provinces of Bihar and Orissa and in the Punjab it was observed that there appeared to be some correlation between low altitude of a land and associated rice cultivation and the occurrence of filariasis (Korke, 1932). The area dealt with in the present investigation possessed different physical features

The blood material for this investigation was derived from different sections of population and in relation to the physiographical conditions of the State

The class of population and the centres at which they were examined were convicts at the Central Jail, Gwalior, patients at the Java Arogya Hospital,

Lashkar, military population at the Brigade Hospital, Lashkar, and convict and hospital population at the Central Jail and State Hospital, Ujjain centre

A fair number of persons examined at the above centres had their residential addresses outside the State areas, and they are therefore shown as coming from their own respective areas in the table of 'Persons with filarial infection in relation to distribution in different areas' in the body of the paper

In all one thousand and forty-one persons were examined. The blood material was taken during night hours of 7 p.m. and 10 p.m. at Gwalior and 8 p.m. and 12 a.m. at Ujjain, and the thick films were treated with recognized methods of staining.

A PHYSICAL ASPECTS OF THE GWALIOR STATE (*Gazetteer of Central India*, 1908)

The Gwalior State is the largest treaty state in the Central India Agency, with an area of 25,041 square miles, and is composed of several detached portions but may be roughly divided into two, the Gwalior or northern, and the Malwa section. The northern section consists of a compact block of territory, lying between 22°10' and 26°52' N and 74°38' and 79°8' E, with an area of 17,020 square miles. The Malwa section, with an area of 8,021 square miles, is made up of several detached districts, between which portions of other States are interposed (*see Map*).

The State falls into three natural divisions, conveniently designated the plain, plateau, and hilly tract. Briefly stated, the *zilas* (districts) of Sheopur, Tonwar-ghar, Bhind and Gwalior Gud are the plains areas ranging from about 500 to 900 feet above sea-level, with a combined area of 7,763 square miles and an average density of population about 167 per square mile. The *zilas* of Narwar and Isagarh are at the commencement of the Malwa plateau, with a combined area of 7,632 and an average density about 85, and at an elevation of about 1,000 and 1,200 feet.

The Malwa plateau encloses the *zilas* of Bhilsa, Shajapur, Ujjain and Mandasor, with an average elevation of 1,500 feet and a combined area of 8,345 and an average density of about 106. The *zila* of Amjhera is a hilly tract, with a mean elevation of 1,800 feet and an area of 1,301 and density of 74.

Jowar is the principal crop, covering 1,807 square miles of the total cropped area in *khalsa* land, while gram (952), wheat (467), *bajra* (341), maize, barley, *arhar* and rice (66) are also cultivated. The chief crops are thus dry crops.

The climate of Central India is, on the whole, extremely healthy and is influenced by the Indo-Gangetic plain on the north and the lofty barriers of the Vindhya and Satpura ranges to the south. These two parallel ranges give a westerly direction to the winds.

The average rainfall of Central India for twenty-five years ending 1901 is 41.30 to 44.98 inches in the Eastern Section (Satna and Nowgong) and 29.39 to 32.70 inches in the Western Section (Neemuch and Indore).



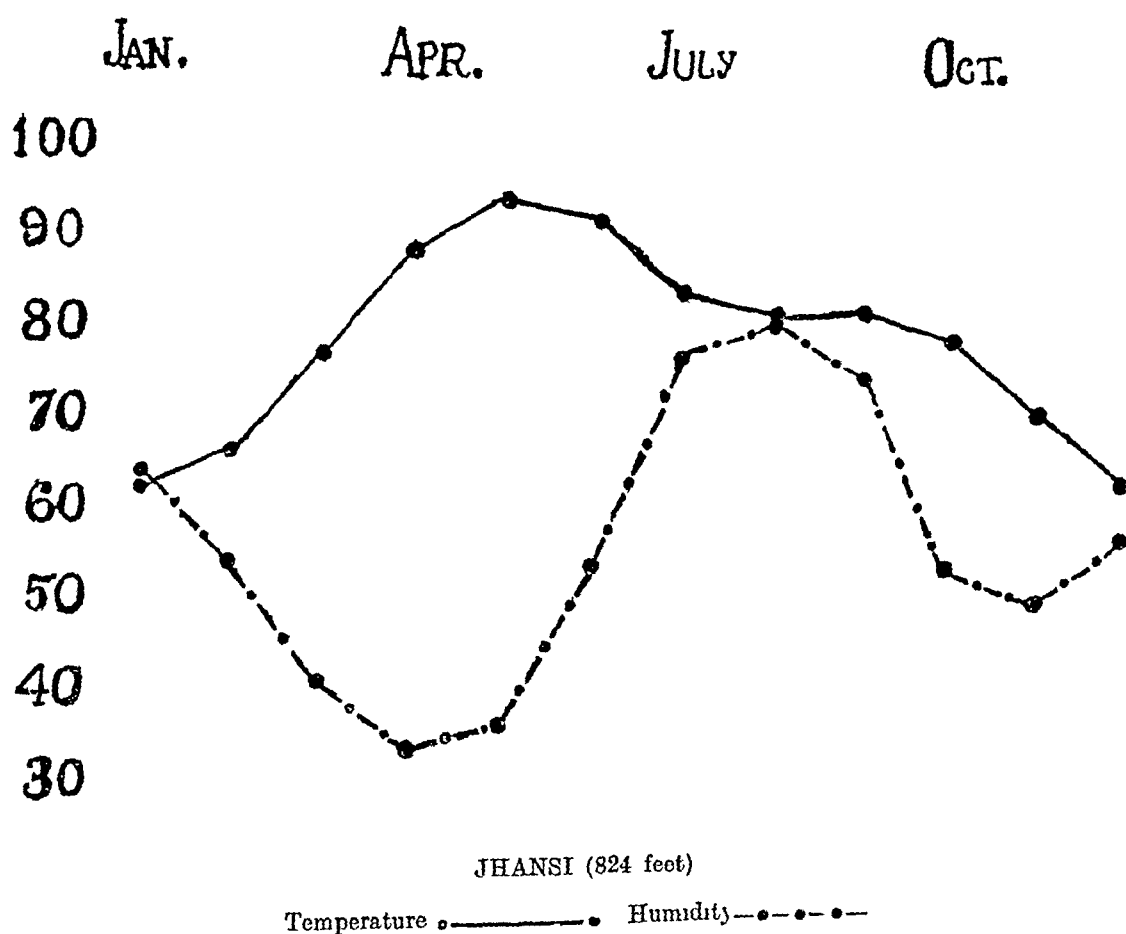
MAP OF CENTRAL INDIA (PART)
GWAJLOR STATE

The variation in the humidity of Central India during the year is also very marked, there being two distinct periods of maximum and minimum. The period of minimum humidity during the summer months occurs in March.

and April on the plateau, and in April and May in the low-lying area, while in both areas November and February are the least humid of the winter months. In August in summer, and in January in winter, the humidity reaches a maximum.

The meteorological conditions of the plains and plateau areas of the Gwalior State should necessarily differ. No meteorological station is maintained on the plains area of the State but the records for Jhansi or Agra (824 feet and 554 feet above sea-level), the adjacent areas situated respectively about 60 and 70 miles south and north of Gwalior, may be taken as representing the conditions in the area. The temperature and humidity conditions of these two areas are practically identical and Graph 1 represents the average temperature and relative humidity

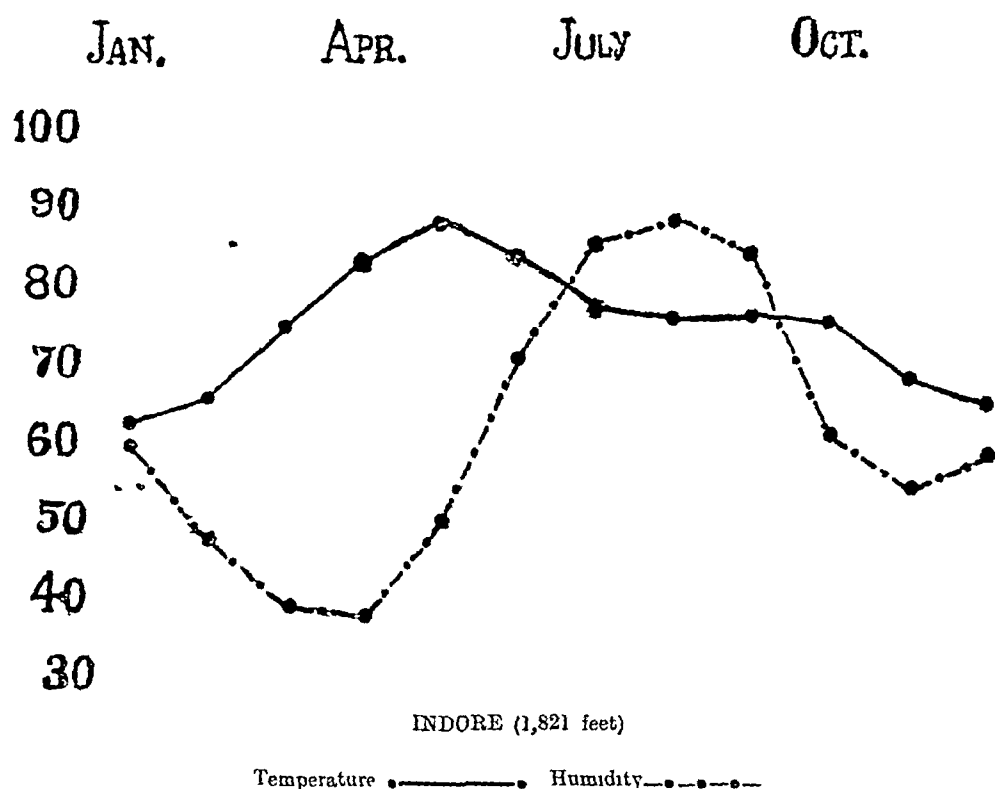
GRAPH 1



of the Jhansi area. The figures from which this and the succeeding Graphs have been prepared were obtained from the Memoirs of the Indian Meteorological Department (1914).

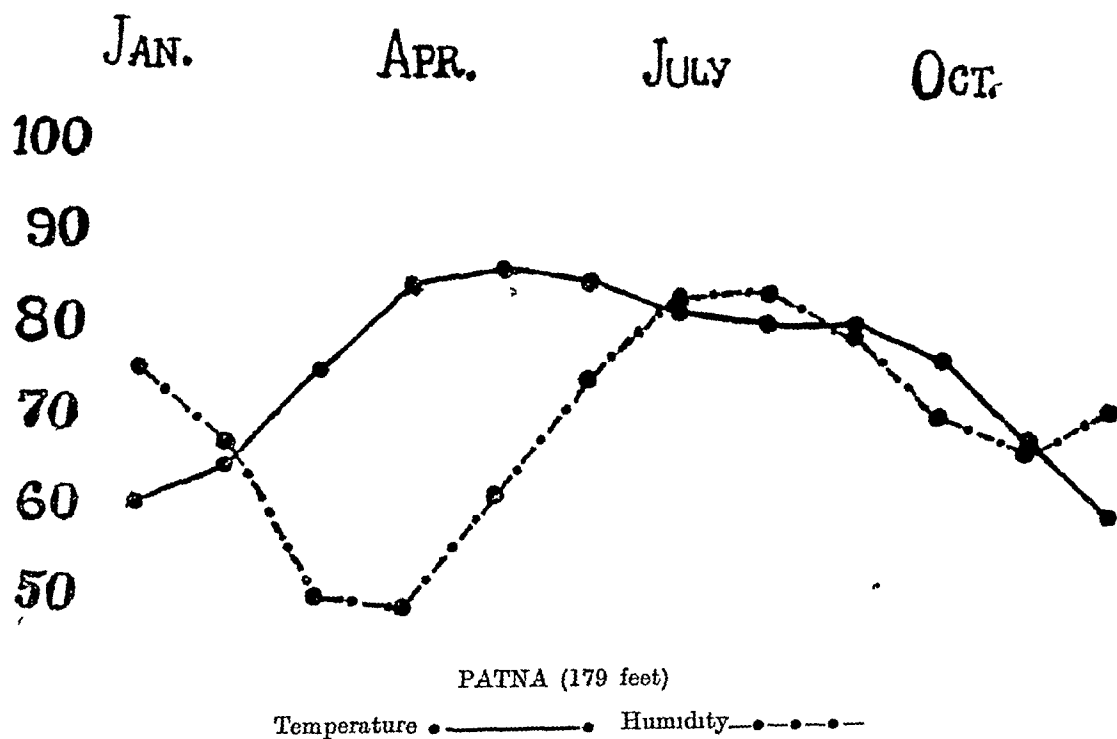
The meteorological conditions of the plateau area of the State are represented by Neemuch and Indore (1,626 feet and 1,821 feet above sea-level), which again are practically identical as regards average temperature and relative humidity and Graph 2 represents these conditions at Indore

GRAPH 2

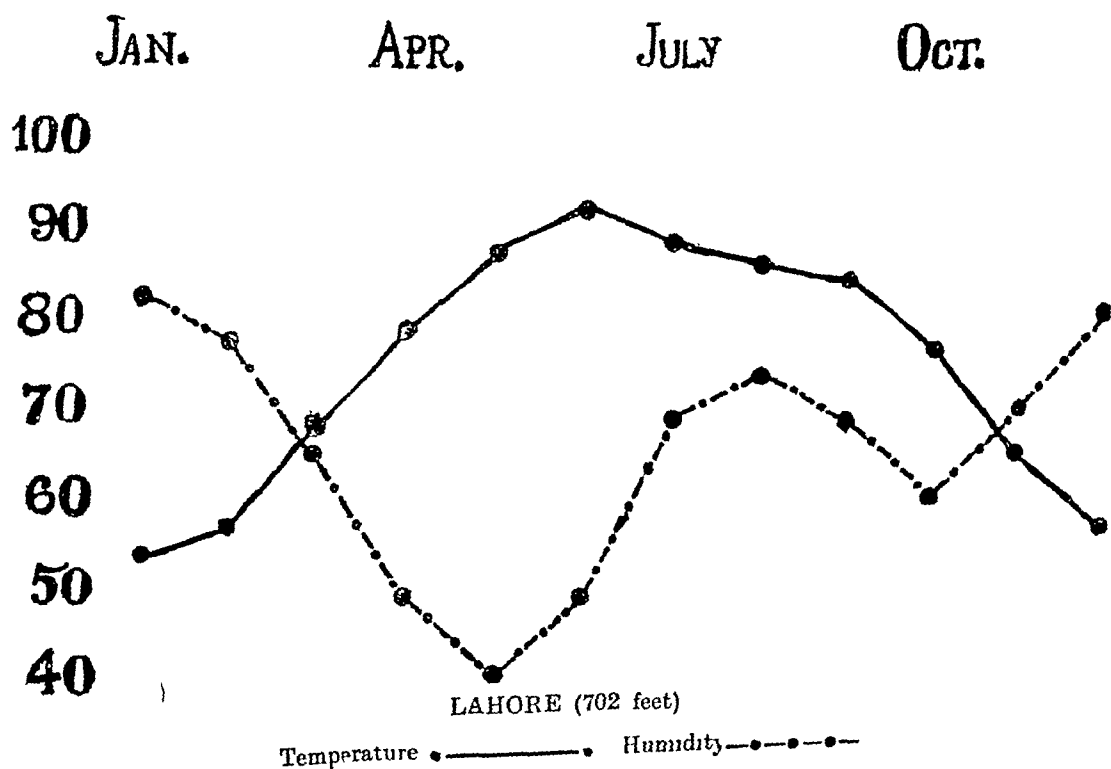


For purposes of comparison of the climatic conditions in Gwahor State with those of other areas in India showing varying incidence of filarial infection, the relative figures of average temperature and humidity of a type plains area in the Gangetic Belt of Bihar and Orissa like Patna (179 feet above sea-level) and a type plains area in the Punjab like Lahore (702 feet) have been given below in Graphs 3 and 4

GRAPH 3



GRAPH 4



B FILARIASIS IN GWALIOR STATE

The blood of one thousand and forty-one persons examined at night between the hours of 7 p m and 12 a m in the Gwalior State area showed microfilaria in 50 persons or 4.8 per cent. The type of microfilaria found in the blood was *Wuchereria bancrofti*.

C ANALYSIS OF THE RESULTS

(1) The following figures show the number of persons with filarial infection in relation to elevation —

(a) Permanent residents of Gwalior State

Elevation	Total examined	Positive	Percentage of positive
500 to 1,000 feet	392	42	10
1,000 to 1,200 feet	170		
1,200 to 1,500 feet	300	2	0.6
TOTALS	862	44	5.1

(b) Persons resident but coming from other areas (other states in the Central India Agency and British areas as U P, C P, the Punjab, N-W F P, Bombay)

Elevation	Total examined	Positive	Percentage of positive
300 to 500 feet	28	3	10
500 to 900 feet	49		
900 to 1,200 feet	41		
1,200 to 1,500 feet	34	1	3
1,500 to 2,000 feet	18	2	11
Over 5,000 feet	9		
TOTALS	179	6	3.3

(2) The following figures show the number of persons with filarial infection in relation to distribution in different areas —

(a) Permanent residents of Gwalior

Tract	District areas	Total examined	Positive	Percentage of positive
Plain	Gwalior Gird (the towns of Lashkar, Gwalior and Morar)	223	36	16
	Tonwarghar	90	1	1
	Bhind	79	5	6
Plateau	Bhilsa and Shajapur	123	2	1.6
	Narwar and Isaghar	170		
	Ujjain, Mandasor and Amjhera	177		
TOTALS		862	44	5

(b) Persons resident but coming from other areas.

General areas		Total examined	Positive	Percentage of positive
From	Indore	14	1	7
"	Other states in C I Agency	76		
"	C P	8	2	25
"	U P	59	3	5
"	N-W F P	5		
"	the Punjab	4		
"	Bombay	13		
TOTALS		179	6	3.3

There is definite evidence of prevalence of filariasis in the Gwalior State proper between the heights of 500 to 1,000 feet, at higher levels as the Malwa plateau is approached the incidence falls rapidly

The Gwalior Gird which includes the towns of Gwalior, Lashkar and Morar shows a very considerable percentage of infection amounting to 16 per cent of the resident population examined. No evidence of filariasis has been found in persons coming from other parts of the Central India Agency except from the Indore area

Immigrants from other parts of India were found infected to the extent of 3.3 per cent. The majority of these came from the Central Provinces and the United Provinces but the percentage shown is based on very small figures

(3) The following figures show the number of persons with filarial infection in relation to age and sex in the Gwalior State —

	Age in years	TOTAL EXAMINED		POSITIVE		PERCENTAGE OF POSITIVE	
		Males	Females	Males	Females	Males	Females
Persons resident in areas below 1,000 feet in elevation	1 to 10	10	1				
	10 to 20	40	10	2	1	5	10
	20 to 30	134	30	9	4	6.7	13
	30 to 40	119	12	9	1	7.6	8
	40 to 50	62	1	9		14.5	
	50 to 60	19	5	3	1	16	20
	60 to 70	23	3	5	1	22	33
TOTALS		407	62	37	8	9	13
Persons resident in areas above 1,000 feet in elevation	1 to 10	1					
	10 to 20	28	1				
	20 to 30	191	1	2	1	1	
	30 to 40	154		1		0.6	
	40 to 50	128		1		0.7	
	50 to 60	51					
	60 to 70	17					
TOTALS		570	2	4	1	0.7	

(4) The following figures show the number of persons with filarial infection in relation to class and type of population —

Type of population	HINDUS			MOHAMMEDANS		
	Total examined	Positive	Percentage of positive	Total examined	Positive	Percentage of positive
Convict	608	5	0.8	58	1	2
School	19	1	5	1		
General—						
Males	97	9	10	16	2	12.5
Females	60	9	15	3	1	33
Military	141	12	9	37	10	27
TOTALS	925	36	4	115	14	12

N.B.—In addition to the above total one Christian female was examined showing negative result for filariasis

(5) *Average number of microfilaria in 20 cmm quantity of peripheral blood per slide*

The embryos of *bancrofti* in 50 positive slides varied from 1 to 50, giving an average of 12.4 per slide in positive cases

(6) *Signs and symptoms*

Systematic record of the signs and symptoms of filariasis in the persons whose blood was examined was not made but from the observations in the wards of the hospitals fugitive swellings and permanent cedemas of the extremities, affections of tunica vaginalis, and chyluria were not infrequently met with, especially in the Gwalior Gird area. As regards the Malwa plateau, the Senior Medical Officer, Civil Hospital, Ujjain, writes to me to the following effect: 'We have had only three cases of filariasis during the three years, only one of which showed microfilaria in his blood, while the others were diagnosed clinically. Only two of these were from Gwalior and one from Orissa. I think my report is valuable to you only from its negative side.'

The incidence of filarial infection in the Gwalior plains area at an elevation of between 500 and 1,000 feet is similar to that found in areas of the Gangetic Belt of Bihar and Orissa at an elevation of 200 to 400 feet, the respective figures being 9.6 per cent for Gwalior State and 9.9 per cent for the area referred to. The absence of infection at the higher levels of the area under survey (over 1,000 feet) correspond to a similar finding at the same level in the Punjab (Koike, 1930a, 1930b, 1932)

A marked contrast exists between the physical, climatic and crop conditions of the infected area of Gwalior State and the Gangetic Belt of Bihar and Orissa, although the incidence of infection in these two areas is almost the same

The endemic area of the Gwalior State is at an elevation of 500 to 1,000 feet above sea-level the country being dry and arid, while the Gangetic Belt at a lower elevation (200 to 400 feet) is damp and moist. The area in Gwalior State is subject to extremes of temperature, while the Gangetic Belt of Bihar and Orissa showing an equal filarial incidence is more moderate in climate and more humid. The former area is a land of dry cultivation, while in Bihar and Orissa rice is extensively cultivated under water. These differences in physical, climatic and crop conditions are striking and would serve to correct an impression, which has been conveyed by previous surveys, that an association of filarial infection with moist humid climate and low-lying rice growing areas is an essential one.

The contrast between the incidence of filariasis in the plains area of Gwalior State and in the Malwa plateau is definite, and the absence of infection in the latter area would indicate that an investigation of the epidemiological factors in the two areas might afford an opportunity of determining the influence which various factors may have on the spread of infection.

As far as general knowledge regarding the presence of *Culex fatigans* goes, the mosquito has an almost universal distribution in India, and yet there are wide areas also that are free from filariasis and the factors affecting its efficiency as a vector of the disease will probably only be determined by a study along such lines, contrasting also the conditions found in other areas of which detailed surveys have been made.

(C) CONCLUSIONS

1. There is comparatively a high prevalence of filariasis in the Gwalior State proper between the heights of 500 to 1,000 feet, but the incidence decreases as higher levels are reached. The Gwalior Gird area shows an infection of about 16 per cent.

2. The type of microfilaria observed was *Wuchereria bancrofti*. An average number of microfilaria of 12.4 per slide was found in positive cases.

3. In the case of males and females age-groups from 20 to 30 years upwards in persons resident in areas below 1,000 feet in elevation showed the highest incidence. The highest incidence shown is in the 60 to 70 years group, being 22 per cent and 33 per cent in males and females respectively. The numbers in the group are, however, small.

4. The area surveyed in which endemic filarial infection was shown to exist presented marked differences in physical, climatic and crop conditions from other areas in India showing a similar percentage of infection.

ACKNOWLEDGMENTS.

I have received the utmost courtesy and willing help from the officials of the Gwalior State. It will be beyond the scope of my acknowledgment, if I were to mention names individually and who have really helped the investigation. The Honourable Home Member, the Chief Medical Officer and Sanitary Commissioner, the Physician, Jaya Arogya Hospital, and his staff, the Jail, School and Military

Authorities at Gwalior and the Senior Medical Officer and his staff and the Jail Authorities at Ujjain have all shown keen interest in the survey and I am highly indebted to them for their very kind and ready help. Finally, I have to thank Mr C W. C. Carson of the Gwalior State through whose kind help I was able to undertake the investigation in that area. My thanks are also due to Captain P J Barraud of the Malaria Survey of India for supplying me information regarding the distribution of *Culex fatigans*.

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PHOTODYNAMIC ACTION OF METHYLENE BLUE ON FIXED RABIES VIRUS

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RECENT work on the photodynamic action of methylene blue on bacteriophage (Clifton, 1931) has revealed the interesting fact that this dye, even in extreme dilutions, has the property of inactivating certain strains of bacteriophage when the mixture of methylene blue and bacteriophage is exposed to light, but is quite inert when the mixture is kept in the dark

Clifton showed, further, that the inactivating effect was probably due to oxidation of the methylene blue as the reaction did not take place *in vacuo* or in an atmosphere of nitrogen

Perdrau and Todd (1933a) repeated and extended Clifton's studies with the same and other strains of bacteriophage. They were able to confirm his observations as to the sensitivity of certain phages to the photodynamic action of methylene blue and as to the necessity for the presence of oxygen

They also proved that the inactivating property of visible light is only possessed by light of certain wave-lengths and that the interposition of a screening layer of methylene blue of sufficient depth prevented the inactivation

A further important finding of these workers was that the addition of the homologous living bacterium to its bacteriophage greatly increased the resistance of the latter to the photodynamic action of the methylene blue, while the addition of killed suspensions of the same organism had no such effect

In a later publication the same workers (Perdrau and Todd, 1933b) extended their observations to the photodynamic action of methylene blue on various filterable viruses

The results obtained by them were almost as clear cut as with bacteriophage and encouraged us to repeat their work with the virus of rabies with the object of

adding one item to our knowledge of this interesting phenomenon and in the hope that some practical application of the principle might be made

EXPERIMENTS WITH THE PARIS STRAIN OF RABIES FIXED VIRUS

Most previous work on this subject has been carried out with filtrates of the bacteriophage or virus concerned, although Perdrau and Todd, in the case of canine distemper, used a centrifuged suspension of ferret's spleen. The effect of using filtrates is to ensure that in the material subjected to the photodynamic action of methylene blue there shall be no gross particles the interior of which might either be screened by the intervening tissues from the action of the light used in the experiment or insufficiently penetrated by the methylene blue solution.

In the experiments with rabies fixed virus performed by us we decided not to use filtrates because our experience has been that filtrates through the usual candles give by no means uniform results and that it is not infrequent to find escapes among animals inoculated sub-durally with filtrates of rabid brain material. It was felt also, that the use of centrifuged emulsions which would contain homologous living cells of the infected brain which was the source of virus would provide the severest test of the inactivating power of the methylene blue on the virus.

In this connection it has been shown by Perdrau and Todd (1933a) that, whereas the presence in the mixture of living cells of the tissue from which the virus was derived gave the latter protection in most cases, even after comparatively prolonged exposure to light, yet, the presence of dead cells and of cells from normal animals gave practically no protection.

In our experiments therefore where centrifuged suspensions of rabid brain were used, the optimum conditions for protection of the virus from the photodynamic action of the methylene blue were present.

MATERIALS AND METHODS

The methylene blue used in experiment I was Methylenblau f. Bac. Koch of Dr. G. Grubler & Co., and in the subsequent experiments medicinal methylene blue (source not known) was used. A sheep infected with fixed virus rabies of the Paris strain and killed when in a moribund condition on the ninth day after infection was used as the source of virus. From the brain a one per cent suspension in distilled water was made by grinding in a mortar with sterile sand. This suspension was centrifuged for two minutes in a hand-driven centrifuge in order to throw down the grosser particles of brain tissue. The resulting suspension was distinctly opalescent to the naked eye but, on microscopical examination under a low power, there were no markedly gross particles. Examination with high powers showed an abundance of cellular material.

The light employed by us was daylight. In the first three experiments this was the diffused light entering a well-lighted verandah, but in the fourth experiment the exposure to light was made in the open. On this last occasion the light varied from direct sun rays to a diffused light during the course of the experiment. This unavoidable factor must have resulted in considerable variation in the intensity of light to which the materials were subjected at different stages in the experiment.

TABLE.

Combined figures of four experiments to test the photodynamic action of methylene blue on rabies sized virus

Time in minutes --	2			5			8			10			15			20			25			30			60			Additional controls
	Number of rabbits	Died of rabies	Survived	Number of rabbits	Died of rabies	Survived	Number of rabbits	Died of rabies	Survived	Number of rabbits	Died of rabies	Survived	Number of rabbits	Died of rabies	Survived	Number of rabbits	Died of rabies	Survived	Number of rabbits	Died of rabies	Survived	Number of rabbits	Died of rabies	Survived				
1 10,000 { Exposed to daylight In dark room	3	3	0	2	2	0	2	1	1	1	1	3	1	0	0*	1	0	1	1	0	1	1	0	1	1	Two further control rabbits were infected each with 0.2 c.c. of 0.5 per cent fixed virus emulsion centrifuged for 2 minutes and then exposed to daylight for one and two hours respectively. Both these animals died of rabies, on the 8th and 9th days respectively.		
	3	3	0	1	1	0	1	0	0*	1	0	0	1	1	1	1	1	1	1	0	1	1	0	1	1			
1 20,000 { Exposed to daylight In dark room	3	2	0*	2	1	1	2	1	1	4	0	4	1	0	1	1	0	0*	1	0	1	4	0	3	4	Both these animals died of rabies, on the 8th and 9th days respectively.		
	3	2	0*	1	1	0	1	1	0	1	1	0	1	0	1	1	0	4	0	1	4	0	4	0	1			
1 200,000 { Exposed to daylight In dark room	3	1	0	2	1	1	2	0	2	1	1	3	1	0	1†	1	0	1	1	0	1	4	0	4	0			
	3	1	0	1	1	0	1	1	0	4	1	0	1	0	1	1	0	1	1	0	1	4	1	0	1			

* Animals not accounted for died of other causes

† This animal showed symptoms suggestive of rabies on the 8th and 9th days after infection, and then appeared to have recovered.

The methylene blue solutions were prepared in daylight in concentrations of 1 in 5,000, 1 in 10,000 and 1 in 100,000. The virus suspension was also prepared in daylight.

The methylene blue solution and the virus suspension were mixed in equal volumes in the dark-room.

Into each of three Petri-dishes of 6 inches diameter were poured 50 c.c. of the methylene blue-virus mixture giving a depth of 5 mm. of the mixture. This resulted in the Petri-dishes containing 1 in 10,000, 1 in 20,000 and 1 in 200,000 dilutions respectively of methylene blue. These Petri-dishes were then exposed to the light while similar dilutions of methylene blue and virus were kept in the dark-room to be used on control animals. Further controls were provided for by the use of virus emulsion mixed with an equal quantity of distilled water and exposed to the same light for one and two hours. Rabbits were inoculated sub-durally with the methylene blue-virus daylight suspension and methylene blue-virus dark-room suspension at intervals as detailed in the tables and with the distilled water-virus daylight suspension at intervals of one hour and two hours. The actual infecting dose in all cases was 0.2 c.c. of a 0.5 per cent fixed virus emulsion which had been centrifuged for two minutes, since the original 1 per cent emulsion was in all cases diluted by the addition of an equal amount of the methylene blue, or, in the case of the additional controls, of distilled water.

In making the sub-dural inoculations the syringes and emulsion containers were protected by non-actinic red paper. The results of four experiments are given in the Table above.

DISCUSSION

A study of the Table will demonstrate conclusively the remarkable action exercised on the rabies virus by methylene blue in the presence of daylight.

It will be seen that the methylene blue produced no saving effect after two minutes' exposure to daylight. After five minutes' exposure a certain saving effect commenced with the higher dilutions of methylene blue but not yet with the lower. At eight minutes' exposure a saving effect commenced with the lowest dilution. Ten minutes' exposure appeared to be the critical period for the dose of virus, the quality of the daylight and the dilutions of methylene blue used by us. At this stage there was a complete saving effect in the dilution of methylene blue 1 in 20,000 and a 3 out of 4 saving effect in the dilutions 1 in 10,000 and 1 in 200,000. In all the periods of exposure beyond 10 minutes there was, with one exception at 30 minutes with a dilution of 1 in 20,000, a complete saving of animals, while all the dark-room controls and distilled water-virus daylight controls succumbed to rabies.

So far as the small numbers concerned show it seems apparent that the weaker dilution of 1 in 10,000 methylene blue is less effective than either of the greater dilutions of 1 in 20,000 and 1 in 200,000. It seems probable that the optimum dilution may lie between these two figures, e.g., 1 in 100,000.

The reason why the stronger solution of methylene blue appears to be less effective than the weaker is explained by the experiments of Peiraud and Todd already alluded to, which showed the inactivating effect of a screen of methylene

blue In our experiments the lesser saving power of the strongest solution of methylene blue was due to the screening effect of the upper layers of the methylene blue solution

One interesting point brought out by the results was that the protective action of homologous living cells observed in similar experiments with the viruses of herpes, Borna disease and fowl plague was not evident in our experiments and this fact brings rabies fixed virus into line, so far as this character is concerned, with the viruses of louping-ill and vaccinia

The question as to whether the photodynamic action possessed by methylene blue can be put to any practical application in connection with the virus of rabies is one for consideration and experiments with this object in view are being undertaken by us

SUMMARY AND CONCLUSIONS

1 Rabies fixed virus in suspension is highly sensitive to the photodynamic action of methylene blue even in very low concentrations of the dye

2 This sensitivity is apparently not appreciably reduced by the presence of living homologous cells

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MORPHOLOGICAL STUDIES ON RABIES

Part I

THE SALIVARY GLANDS

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I INTRODUCTION

MOST diseases whose transmitting agent is known, such as malaria or relapsing fever, differ from rabies in respect of the fact that there is no definite and dramatic act of transmission recognized at the time by the sufferer, such as occurs in rabies, where the transmission is effected by the bite of a rabid and savage animal. This very fact of the disease following upon the bite of such an animal pointed inevitably to the assumption that infection was conveyed by the saliva on the teeth of the animal and such an inference was drawn by the earliest investigators. These facts being so, it has seemed strange to us that comparatively so little work appears to have been done on the salivary glands of rabid animals and so much on the central nervous system. This is probably due to the discovery by Negri in 1903 of the bodies bearing his name, a finding which has proved an invaluable method of diagnosis in street rabies and has led to minute investigation of the central nervous system by many workers while the study of the salivary glands has been, comparatively speaking, neglected.

That it has been so neglected, in spite of the studies of a few distinguished workers such as Manouéhan and Viala, is evident from the scanty literature on the subject and from the fact that our study of a large amount of rabid glandular material, both human and animal, has been productive of the discovery of various conditions, which we find constantly present in suitable cases, but which we have not seen previously described. That the neglect to describe some of the

appearances seen by us is really due to lack of study of the glands is obvious from the fact that these appearances are so striking and so definite that they could not possibly be missed by anyone familiar with the normal appearance of salivary glands. As will be seen in the description given below some of the appearances described by us we are able to explain and even to reproduce in known non-rabid animals, while others appear to be normally present but are exaggerated in cases of rabies. It is in the hope that the description here given of our findings, together with the small amount of previously recorded work on the salivary glands in rabies, will lead others to repeat and extend our work, and to criticize it or confirm and enlarge upon it, that we have placed on record our observations which are admittedly still far from being a complete exposition of the changes found in the salivary glands in rabies.

II GENERAL STRUCTURE OF THE SALIVARY GLANDS

The essential structure of all salivary glands consists of a series of acini held together by loose but strong connective and fibro-elastic tissue. The acini pour their secretion into ducts which, by anastomosis with other similar ducts, gradually increase in size and terminate in one or more main excretory ducts.

While this is the general character of all salivary glands, minor differences are present both as regards structure and function according to the animal being considered. These need not be entered into here but a short consideration of the histology of salivary glands, limited to those essentials only which come in for consideration in our observations, is necessary to a full appreciation of the description we have given of our findings in the salivary glands of rabid animals and man.

The largest salivary glands are the parotid and sub-maxillary and these chiefly are the subject of our present study.

In most mammals there is a general agreement as to the anatomical distribution of the glands but there are minor histological differences and those presenting themselves in dog, man and monkey, which are the animals we have chiefly studied, will be dealt with briefly.

The parotid gland

In each of these animals the parotid gland is situated superficially in the region anterior to the ear. It is a comparatively thin layer of gland tissue and sends irregular ramifications in all directions from this central area.

Histologically it is a pure serous gland consisting of acini lined with pyramidal cells and having more or less centrally placed sub-spherical nuclei. The cells, when suitably fixed and stained, contain fine granules of secretion. The lumens of the acini are comparatively small under most conditions, the cells more or less filling up the acini.

The secretion granules are very sensitive to various reagents and are often completely dissolved during the processes of fixation, hardening and staining and in such cases the cytoplasm has a marked reticulated appearance.

The sub-maxillary gland

This is a more compact gland than the parotid and lies chiefly under cover of the lower jaw but also envelops its outer surface. This is a mixed gland in that it is composed of both serous and mucous acini and of mixed acini. In the human sub-maxillary and, to a lesser extent, in that of the monkey (*M. rhesus*) the mucous alveoli are in the minority, forming about one-quarter of the entire gland but in the dog the mucous alveoli greatly preponderate.

The mucous cells as usually seen in sections hardened in alcohol are clear and swollen, being distended with mucigen with the nuclei situated near the basement membrane of the acinus.

In the mixed alveoli the serous cells lie next the basement membrane and form crescentic marginal areas called demilunes or crescents of Gianuzzi.

The accessory salivary gland

This is the name which we have given to the glandular tissue which is found surrounding the duct of the parotid gland. The cells of this glandular tissue are essentially serous in type but differ slightly from the typical serous cells of the parotid. They appear to be somewhat more finely granular and to have less tendency completely to fill the lumen of the acinus. Closely associated with the glandular tissue in this situation is much lymphoid tissue which may, with the naked eye, easily be mistaken for part of the salivary gland.

III MATERIAL AND METHODS

Fortunately the material which forms the subject of our observations has been limited only by our capacity to deal with it. It has consisted of naturally occurring rabies in man, dogs and other animals, rabies induced in dogs and monkeys by sub-inoculation into the muscles of the neck of material obtained from the brain of rabbits which had undergone a first passage from a naturally occurring street virus, and rabies induced in dogs and monkeys by inoculation into the neck muscles of naturally occurring street virus. The street virus used for inoculation was sometimes that obtained from the brain and sometimes that obtained from the salivary glands. In all our experiments in this series we have consistently inoculated animals into the muscles of the neck, especially avoiding the intracerebral, spinal, or subdural routes, in order to reproduce more nearly the method of natural inoculation by the biting animal.

By all these methods our findings have been identical and we consider that the disease as induced in first and second passages, at least, is essentially that which occurs in naturally infected animals.

The question of suitable fixatives and staining methods and the proper combination of these is an important one.

It is not necessary for us to go into a detailed description of all the methods used by us in preliminary work and we shall only indicate our main conclusions.

As regards fixatives we found that those containing perchloride of mercury were incomparably the best for the stains chiefly used by us and that the more rapid and penetrating the action of the fixative the better the results. After trying

various reagents the one which we came to employ as a routine was Sansom's modification of Carnoy's fixative. The formula is as follows —

Absolute alcohol	65 c c.
Glacial acetic acid	5 „
Chloroform			30 „
Mercury perchloride to saturation			Fix for half an hour

The fixative should preferably be prepared the day before use and should never be kept for more than a week or ten days.

Tissues placed in this reagent are adequately fixed in about half an hour or less and should not be left in much longer as the fixative is probably the most penetrating known and prolonged immersion of objects will render them so hard and brittle as to make subsequent cutting a most difficult matter.

The tissues should be removed from the fixative into absolute alcohol strongly coloured with iodine for 24 hours or overnight then into absolute alcohol which should be changed several times during 24 hours. The subsequent procedures are those normally adopted for embedding in paraffin but double embedding in celloidin and paraffin may be resorted to if desired.

The two staining methods of choice and those used in most of our work were the methods of Mann and iron-hæmatoxylin, the latter combined, when necessary, with a counter-stain such as neutral red or a special light green (not S. F.). When a counter-stain to iron-hæmatoxylin is desired it is best applied in the manner indicated by the senior author in a previous publication, viz., by dissolving the counter-stain in carbol-xylol (Shortt, 1923). The stained film is passed from the absolute alcohol stage into the carbol-xylol mixture containing the counter-stain, then into pure xylol and mounted in Canada balsam.

In the description of our findings which follows we have confined ourselves, so far as possible, to those points which appeared to us to differ from the appearances seen in normal glands as it is taken for granted that the interested reader will be familiar with these. When we use the term 'normal gland' we mean a gland in the varying conditions in which it may appear in a healthy animal within the limits of its physiological activities. We do not wish to imply that all the conditions we describe are only found in cases of rabies, in fact such is not the case as our subsequent descriptions will show, but merely that the conditions described are such as, in our experience, do not occur in a gland in the ordinary course of its functional activities, and are regularly or frequently seen in the glands of animals suffering from rabies.

IV CONDITIONS OBSERVED IN THE SALIVARY GLANDS OF THE DOG IN RABIES

The dog has been chosen for description as being the animal most typically affected by this disease. Monkey and man have been similarly studied by us and, as the appearances seen are in the main very similar in all these animals the two latter will only be briefly mentioned after the dog has been dealt with in order to bring out differences in detail where these exist.

It should also be pointed out here that our description is a composite one based on the examination of very large numbers of animals, so far as dog and monkey are concerned, although only on a small number of humans, and that the whole of the appearances described are seldom to be met with in one and the same animal. A consideration of the explanations we have offered to account for some of the appearances seen will show that this is inevitable since many of them depend on the state of physiological or pathological activity of the glands at the time our material was taken. Another factor leading to variation in results is the fact that all parts of any particular gland do not show the same appearances. Thus, sections cut from one area might show any of the characteristic appearances to be noted later while in those cut from another part these might be entirely absent.

(a) *Findings in the earlier stages of infection before the onset of symptoms of rabies*

If artificially infected animals are sacrificed before the onset of symptoms it is necessary that only those should be considered the salivary glands or brain of which produce rabies when inoculated subdurally into test rabbits. This is the only way of being sure that one is dealing with an animal in the incubation period of rabies and only such have been included by us in the series we have examined for the purposes of the present section.

In these early stages the glandular elements show little, if any, changes from the conditions found under varying conditions of activity in normal glands. The only definite finding which was met with frequently, although not invariably, was the presence in preparations stained by Mann's stain of small red or magenta-coloured granules in the interacinar supporting tissues of the parotid and sub-maxillary glands (Plate XXIX, fig. 10). These granules varied in size from about 1.5μ in diameter to the downward limit of the resolving power of the microscope. The larger forms are seen to be circular and regular in outline. The majority of these chromatic elements appeared to be lying in endothelial cells which had a very close connection with the endothelial lining of capillaries in the interacinar connective tissue and in some cases appeared actually to be present in the capillary endothelium itself (Plate XXIX, fig. 10). In other cases no direct relationship to capillary endothelium was apparent and they appeared to be widely scattered among the loose connective tissue fibres of the interacinar spaces (Plate XXIX, fig. 10).

The granules are stained equally well, but of a black colour, by iron-haematoxylin but the finding of them in preparations made by the latter method should always be confirmed by using Mann's stain where the differential coloration is of great assistance.

(b) *Findings in the earlier stages of infection after onset of symptoms*

Once symptoms have manifested themselves the disease has advanced to a stage where appearances in the salivary glands become more constant and more characteristic. These appearances will be considered *seriatim* but it should be pointed out that whereas all the appearances to be now described may be present in one and the same case there will be other cases where only some, or even only one, of the conditions described is present. This dissimilarity is probably more apparent than real because we have found that in any one gland the conditions described are

not present uniformly throughout the organ and that, while one area of a gland may present profound changes in structure and function, another area in the same gland may be apparently unaffected. This feature is characteristic of all stages of the disease and may be as marked in the later stages as in the earlier.

1 *Small red or magenta-coloured granules in the interacinar connective tissues*—Many of these were identical with those described in the previous section and require no further mention.

2 *Larger granules in endothelial cells*—In some dogs, there were present in the interacinar tissues granules taking the same coloration with Mann's stain as those described in the previous section but larger in size and contained in cells of endothelial type, sometimes somewhat elongated like primitive connective tissue cells (Plate XXIX, fig. 12). The larger cells containing these granules may have been the macrophages which are normally present in small numbers in these situations (Plate XXIX, fig. 11).

3 *Eosinophil intra-cellular bodies in the interacinar connective tissues*—These forms were found to be present both in the parotid and sub-maxillary glands. They consisted of eosinophil granules, spherical in shape, which were packed closely in a cell sometimes compact and sharply defined (Plate XXIX, fig. 1), sometimes considerably elongated and with its limits less sharply defined (Plate XXIX, figs. 2 and 3). The nucleus of the cell is often almost completely obscured by the closely packed eosinophil granules but, when apparent, is roughly oval in shape and often stains rather faintly with Mann's stain. Occasionally, when the nucleus is eccentric in the cell, it is pressed to the periphery by the granules and markedly flattened (Plate XXIX, fig. 5).

The eosinophil bodies in some cases give the impression of bursting out of the containing cell to scatter in the surrounding tissues (Plate XXIX, figs. 2 and 3). The direction of this scattering is usually in the long direction of the fibres of the connective tissue and it is possible that the appearance is deceptive and that the apparently free bodies are contained in tenuous prolongations of the enclosing cell which are insinuated between the various planes of connective tissue fibres.

The size of the eosinophil granules varies from extremely minute forms of about 0.8μ in diameter to forms of 4μ in diameter, with various stages between these limits. The tendency is for any one group to contain granules of approximately the same size and the gradations in size of the component granules of different groups leads us to suppose that the groups of larger granules are produced by the growth of the groups of smaller granules.

The shape of the granules was always spherical and they stained less intensely than the magenta-coloured granules previously described.

The nature of these eosinophilic bodies is not known and we have not seen any description of them but we find that they represent a normal, although uncommon, constituent of the interacinar connective tissues, being really components of the containing cell and that these cells appear to be increased in number as a reaction to the rabic infection, or possibly, merely to the increased salivary activity.

4 *Granules in the cells of the serous acini or serous cells of mixed acini*—These granules take the form of a 'peppering' of the cells of serous acini with red or magenta-coloured granules when sections are stained by Mann's stain (Plate XXX, fig. 23) and with black granules when stained with iron-haematoxylin. This

peppering may consist of comparatively few granules widely separated or a cell or cells of the acinus may be filled with closely approximated granules. The granules are seldom more than 1.4μ in size and the smallest forms reach the limits of the resolving power of the microscope. The general effect in any one cell, however, is of irregularly shaped granules approximately equal in size. The granules are distributed throughout the cell but with a tendency to be somewhat more scanty in the area between the nucleus and the basement membrane. Where a whole acinus or most of its cells are affected the general effect is a thick grouping of the granules in the centre and a gradual thinning out towards the periphery (Plate XXX, fig. 17) but this effect may be reversed where the granules are in the demilunes of Gianuzzi in a mixed acinus (Plate XXX, fig. 23). There does not seem to be any marked change in the nuclei of the cells containing them as compared with those which are free from them but the cytoplasm looks somewhat rarefied and tends to stain more faintly.

An examination of the salivary glands of normal monkeys and dogs in a state of ordinary physiological activity may show an occasional granule in a serous cell but nothing corresponding with the aggregations we are here describing.

It must be remembered that by using the technique described by us the ordinary secretion granules of the salivary cells are dissolved away.

The question therefore arose as to whether these granules were normal products of the cell exaggerated in amount by a hyper-stimulation of secretory activity or were actual organisms or, possibly, inclusion bodies resulting from the presence of the rabies virus. To settle this point we selected a monkey, as being the animal showing these granules most characteristically, and inoculated it with $1/5$ grain of pilocarpine nitrate. The animal was sacrificed forty-five minutes afterwards. Although it was salivating freely this was not very evident because, after ejecting a quantity of frothy saliva soon after the drug was injected, it continued to swallow the rest. The serous cells of the parotid gland in this monkey contained large numbers of granules similar to those found in the rabid animals and it seems probable therefore that they are merely the coagulated product of the secretion poured out by hyper-stimulated serous cells. One curious fact which should be mentioned is that the areas showing this reaction were not present in all parts of the gland and even in individual sections only some parts were affected. The tendency was for the areas around the edges of the sections to be most affected and we consider this to be due to the fact that the periphery of the tissues placed in the fixative were acted upon more energetically by the fixative than the interior which could be reached by it only after a certain amount of dilution or alteration in the intervening tissues.

5 *Eosinophilic vacuole-like structures in the cells of serous acini*—These structures are present in serous acini only. Their appearance is more fully described later [section (c1)] and they are only mentioned here because they make their first regular appearance at this stage.

6 *Inclusions in the capillaries*—In the capillaries found in the interacinar connective tissues there may frequently be seen rounded coccus-like bodies. These may occur singly or in pairs like diplococci but, more typically, are found in aggregations of greater or smaller size. They may number fifty or more in the

larger aggregations and may extend along considerable lengths of the capillaries. They vary in size from minute forms 0.7μ in diameter to forms 2.6μ in diameter. Larger forms than these may also be seen. The average diameter is about 1.2μ . In outline the bodies are regular and are usually spherical or of a broad ovoid shape.

In specimens stained with Mann's stain they take on an intense red stain and are very striking objects (Plate XXIX, fig 13). In specimens stained with iron-haematoxylin they are jet black but, on being differentiated further, sometimes show local areas where the process of differentiation seems delayed. This may sometimes even give a single body the effect of bipolar staining. A common situation to find a large number of these bodies is at places where the capillaries branch (Plate XXIX, fig 14) but any site may contain them. These bodies, which are not confined to the capillaries in the salivary glands, but may also be found in those of the brain, may be most conveniently demonstrated in thick smear preparations of the brain. These should be made either from the hippocampus major or from the cortex or, indeed, any other part of the brain. In such preparations the capillaries, being more resistant structures than the rest of the brain matter, are well shown and the bodies may easily be found in stained preparations.

Koch and Rissling (1910) have described coccoid bodies in the capillaries and veins of the brain and salivary glands and it is probable that these are the bodies they refer to. They maintain that they are only found in cases of rabies.

As regards the actual situation of the bodies they sometimes give the appearance of being contained within the cells of the capillary endothelium but we believe, from a close study of them, that they are actually in the lumen of these vessels. This assumption must be considered correct if our interpretation of their nature is accepted.

A careful consideration of them has led us to believe that they are actually blood platelets. This would imply their presence in normal tissues and, so far as brain substance, where suitable smears are easily made, is concerned, we have found them to be present. The fact that they appear to be much fewer in number in normal brain tissue than in rabid brain tissue is no argument against their being a normal element, and their relative greater frequency in rabid material may reasonably be explained by some alteration in the blood picture produced in response to infection with the rabies virus or its possible toxin.

Their smooth and regular contour compared with that usually seen in the case of platelets air dried in blood smears is probably due to the fact that they have been fixed *in situ* in the capillaries and so have not been exposed to the disruptive surface tension of a drying blood smear.

(c) Findings in those cases where the symptoms of rabies have persisted
for 2 to 3 days at least

In most of our experiments we tried as far as possible to use material from living animals for examination in order to prevent any fallacies due to post-mortem changes in the tissues. In the case of both monkeys and dogs we found that it was seldom safe to leave an animal beyond the third day after the appearance of symptoms, although we have known a monkey to survive for six days and it was still alive when taken for post-mortem and would probably have lived for at least seven days.

In the case of certain very powerful viruses we found that monkeys frequently died without showing any, or only the most transitory, symptoms of rabies. In these cases it was usual to find no Negri bodies in the brain, or bodies too small to be definitely identified as such, but the brains on passage to rabbits produced typical rabies with Negri bodies in the latter.

For the purposes of our description such cases would be classified as early cases in so far as the production of visible lesions was concerned and they are only mentioned here in order to draw attention to the property of certain very powerful viruses of killing an animal without the previous production of symptoms of rabies and without the characteristic production of Negri bodies, an indication of the importance of performing the biological test in such cases.

1 *Eosinophilic vacuole-like structures in the cells of serous acini*—In certain dogs, less commonly in monkeys and man and never to the same extent, are found spherical vacuole-like structures in the cells of the serous acini (Plate XXX, fig. 22). These have already been mentioned in a previous section (65) but, as they reach their most characteristic development in the more advanced stages of rabies their description has been deferred to this stage.

These forms are confined entirely to serous cells and, when found in the submaxillary gland of the dog, are found only in serous acini and in the serous demilunes of Ginzuzzi in mucous acini (Plate XXX, fig. 19).

The vacuole-like structures vary in size from minute forms, which are to be distinguished from the granules described under (64), up to forms $5\frac{1}{2}\mu$ in diameter. They are perfectly spherical in form, stain a deep crimson or magenta with Mann's stain and take the iron-haematoxylin stain very intensely.

They are never numerous in a cell, especially when large, but three or four large forms may be present in a single cell. They may occupy any position in the containing cell.

As regards structure they tend to stain very uniformly and, in the case of iron-haematoxylin preparations, differentiate uniformly.

In certain cases, however, many of the structures are found to exhibit one, two, three or even more internal bodies looking like vacuoles within the vacuole (Plate XXX, fig. 20). These internal bodies may be, when single, as much as half the diameter of the enclosing vacuole but are usually considerably smaller.

As regards the nature of these vacuole-like structures we are no longer in doubt. At first we were unable to find them in normal controls. We therefore starved three dogs for a period of four days but allowed them access to water. One of these dogs was then sacrificed and showed no vacuoles in the cells of the salivary glands.

The second dog was shown some food but without being given it and then, after an interval of ten to fifteen minutes, sacrificed. This also showed no vacuoles in the cells of the salivary glands.

The third dog was given $1/5$ grain of pilocarpine nitrate subcutaneously. It soon began to salivate very freely and was sacrificed half an hour after the inoculation. The serous cells of the salivary glands of this dog showed typical vacuole-like structures indistinguishable from those seen in rabid dogs. We subsequently kept a dog and a mouse continuously under the influence of pilocarpine for three days and then examined their salivary glands by biopsy. The dog showed an

exaggerated form of the condition just described with very numerous and sometimes very large vacuoles twice the diameter of the largest forms seen in rabid dogs, while the mouse showed no vacuoles whatever. The vacuoles, therefore, are evidently formed when the serous cells are stimulated by a toxin whatever its origin, to hyperactivity and have no necessary relation to the presence of a living virus. It is presumed that the vacuoles are formed by the coalescence of droplets of secretion and that the absence in normal dogs is due to the fact that the abnormal degree of stimulation to the serous secreting cells necessary to produce them in numbers is seldom present under natural conditions. We have since seen a very few of these vacuole-like structures in one dog, presumably normal, but they were small and few in number and probably were associated with a condition of hyper-secretion due to ingestion of food. The cells containing the bodies were not otherwise altered from normal cells as regards their staining reactions and this is only to be expected if we are right in considering the phenomenon to be merely a normal, although exaggerated, response of the cell to powerful stimulation.

2 *Eosinophil intra-cellular bodies in the interacinar connective tissues*—These bodies have already been mentioned under section (b3) and they are to be found in all stages as regards the size of the eosinophil granules. In the later stages of rabies they have been seen by us in dogs, monkeys and man, the largest forms having been seen in the latter (Plate XXIX, figs 6 and 7). They are never numerous in any case or at any stage of the disease. The typical form of the eosinophil granules is, as already stated, spherical but in one instance a form was seen with navicular granules very uniform in size and shape. The significance of this form is not known, it occurred in a dog (Plate XXIX, fig 4).

3 *Multiple aggregations of irregularly ovoid bodies in the serous acini*—In a certain number of dogs which had shown symptoms of rabies for two or three days before death or biopsy the serous cells of the salivary glands showed a very characteristic phenomenon.

This consisted in the presence of enormous numbers of irregularly ovoid bodies arranged in an irregular grouping in the serous cells. In some cases the cells of the entire acinus were crowded with these bodies (Plate XXX, fig 21); in other cases only a single cell in an acinus might contain them while all stages intermediate between these extremes were to be met with.

The size of these bodies was extremely variable. Minute forms 0.5μ in diameter and large forms 2.6μ in diameter were bridged by a complete series of intermediate sizes. There seemed to be no special grouping of the forms by size and one cell might contain forms all of approximately the same size while another might contain forms of many differing sizes. In sections from tissues fixed in Flemming's solution it was noticeable that the bodies were definitely arranged around the peripheries of the sections, a relationship previously noted in connection with the small granular formation described in section (b4) and probably due to the same cause. They were especially abundant where the periphery of the section corresponded with the capsule of the gland or with any other continuous coat of connective tissue forming the surface of the fixed block of tissue.

In tissues fixed by Flemming the ovoid bodies tended to have a rather 'fuzzy' outline and stained, as a rule, a bluish colour with Mann's stain but sometimes had a

pink tinge. In some cases traces of red colour were present in the bodies but the general effect was to present a 'fuzziness' in staining reaction as well as in outline.

In sections stained with iron-haematoxylin and counter-stained with neutral red a clearer picture was presented. The neutral red stained the matrix of the bodies and many of them presented either a single black-staining granule or a dark irregular mottling which disappeared completely if differentiation was carried far (Plate XXX, fig. 21).

The relationship of these bodies to those found in human cases and their probable nature will be discussed at a later stage when we are dealing with our findings in human cases of rabies.

4 *Granules in the cells of the serous acini* — These are similar to the forms mentioned in section (b4) and require no further description (Plate XXX, fig. 23).

5 *Inclusions in the capillary endothelium* — These are the forms dealt with in section (b6) (Plate XXIX, figs. 13 and 14).

6 *Bacillary, club-shaped and irregular bodies in the cells of the serous acini* — These bodies are met with both in dogs and monkeys and take the form of irregular groups which may have any of the shapes mentioned in the heading to this section. They vary greatly in size and their chief characteristic is their pleomorphism in contrast to most of the bodies which have previously been dealt with. The largest of these forms may measure 2.6μ in their longest diameter and very minute forms are also met with. They have only been seen in serous cells. With Mann's stain they assume a bright red or magenta tint and with iron-haematoxylin a black colour. Their staining reactions are identical with those described for previously mentioned forms which have been interpreted as the result of the fixative on the secretion of the serous cells at some stage in its manufacture and we have no reason to doubt that these forms also are coagulated serous secretion or its precursor.

7 *Fat vesicles* — In nearly all the rabid animals we examined, including man, the serous cells of the salivary glands, especially in the later stages of infection, showed more or less numerous fat globules. We use the term fat in the wider sense of some lipid substance as we are not aware of the exact nature of the globules. The globules were very evident in tissues fixed in Flemming's solution owing to the fact that they were stained a brownish yellow by the contained osmic acid. The globules varied in size from minute forms up to forms 7μ in diameter and in number from one or two in scattered cells to a condition where the histological details of the section were almost obliterated by the size and abundance of the fat globules. They were most abundant, in our experience, in the human cases of rabies. Sometimes the presence of fat was shown in more massive and less regularly globular form.

8 *Negri bodies in cells of nerve ganglia* — Nerve ganglia of the sympathetic nervous system are scattered throughout the salivary glands in the inter-lobular tissues especially near the larger ducts and in these Negri bodies, apparently identical with those found in the brain, are occasionally to be seen but never, in our experience, in the numbers found in the cells of the hippocampus major (Plate XXIX, fig. 9).

9 *Polymorphous bodies in the excretory ducts* — In the excretory ducts of all the types of salivary glands we constantly found present bodies of varying shape and size which stained red or magenta with Mann's stain and black with

iron-haematoxylin There was nothing to suggest that these were other than the coagulated products produced by the action of the fixatives used on the secretion in the ducts (Plate XXIX, fig 8)

V CONDITIONS OBSERVED IN THE SALIVARY GLANDS OF THE MONKEY (*Macacus rhesus*) IN RABIES

In the monkey the forms described in section IV (c) are to be found with the exception of those detailed in sections (c3) and (c6) which were not encountered by us On the other hand the forms to be seen even in dogs differed so much in individuals that only the examination of very large numbers of monkeys would suffice to exclude the occasional occurrence of the less common conditions

VI CONDITIONS OBSERVED IN THE SALIVARY GLANDS OF MAN IN RABIES

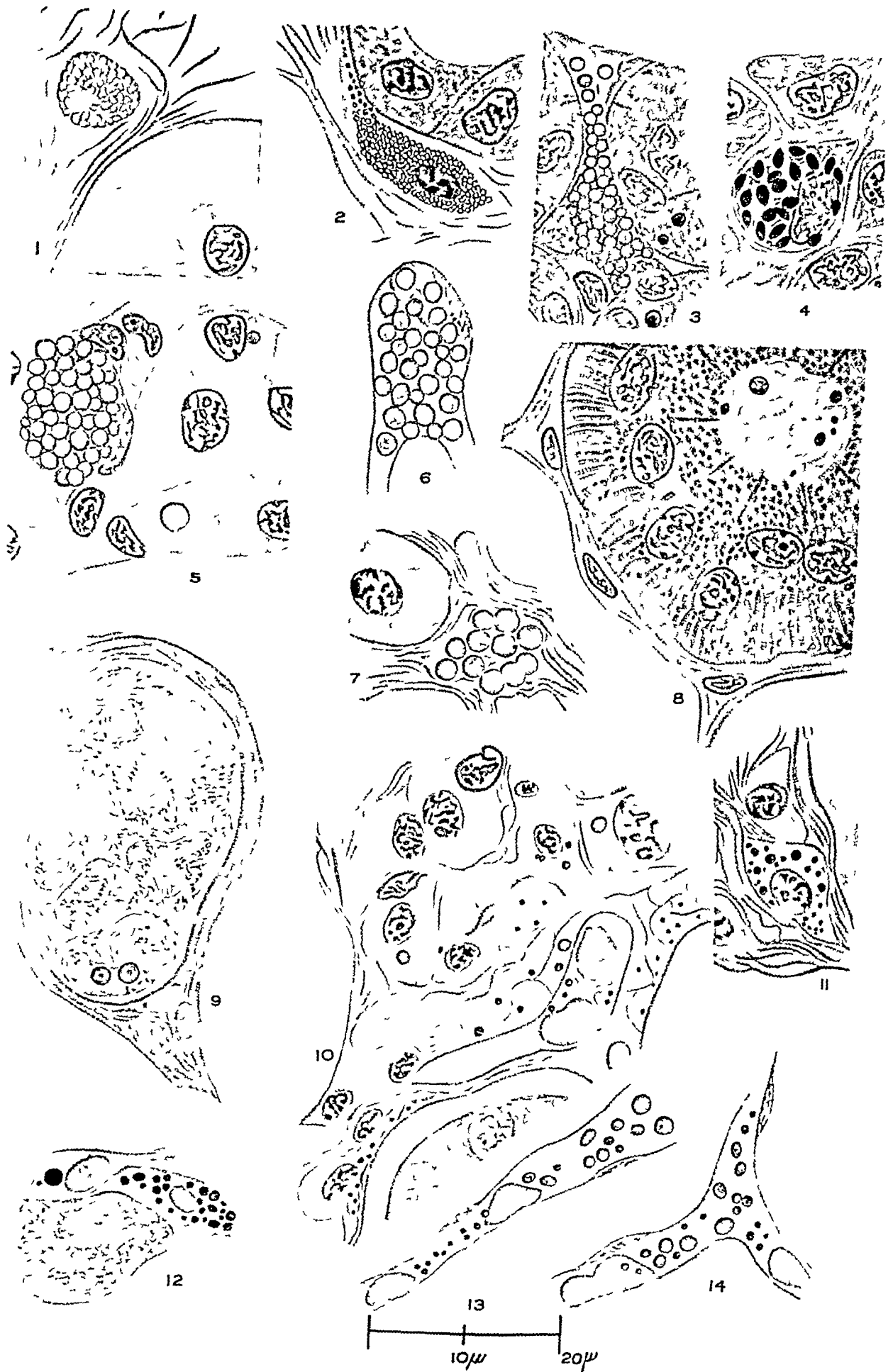
1 *Dichromic ovoid bodies in the cells of the serous acini*

In the case of certain dogs which had shown symptoms of rabies for two or more days before being examined by biopsy we described the presence [section 4 (c3)] of multiple aggregations of irregularly ovoid bodies in the serous acini (Plate XXX, fig 21) In human cases identical structures have not been met with but multiple aggregations of sharply differentiated (in contra-distinction to the 'fuzziness' of those seen in the dog) ovoid bodies have been observed in four out of five consecutive human cases which had received no treatment (Plate XXX, figs 15, 16 and 18) These bodies were present in serous cells only They were sub-spherical bodies of regular contour varying in size from extremely minute forms to forms 2.8μ in their longest diameter In some cases most of the forms present in one serous cell were approximately equal in size while in other cases forms of all sizes were present in the same cell (Plate XXX, fig 16) In some acini only a single cell might contain the bodies while in others every cell might be full of them The appearances here described were only seen in tissues fixed in Sansom-Carnoy fixative and were not brought out in Flemming-fixed material

The staining reaction of the ovoid bodies was most characteristic and striking but differed in different acini

Mann's stain—Typically each body consisted of a bright blue-staining matrix in which was embedded a red or magenta-staining mass which was often eccentrically placed The latter tended to be ovoid but sometimes elongated forms simulating division were seen (Plate XXX, fig 16) The proportions of the red and blue components of the bodies varied considerably sometimes one and sometimes the other preponderating A good idea of the appearance of these bodies will be obtained if one says that, although stained by Mann's stain, they gave the appearance of protozoa stained by Giemsa's stain (When actually stained by Giemsa's stain the characteristic appearance was not obtained) In some acini the groups of bodies, instead of exhibiting the typical dichromic effect of red and blue, stained only red or only blue and in these cases approximated much more closely in appearance to the aggregations described in dogs [section IV (c3)] The same acinus sometimes contained both types or these might even be contained in a single cell

PLATE XXIX

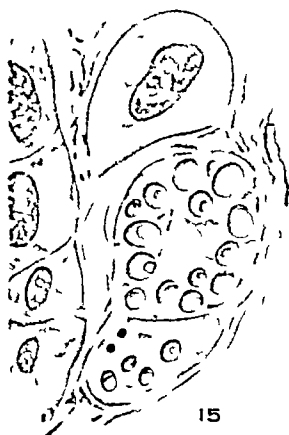


EXPLANATION OF PLATE XXIX

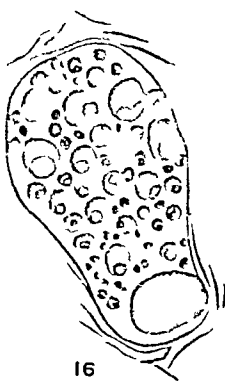
- Fig 1 Eosinophil intra-cellular bodies packed closely in a compact sharply defined cell From dog Mann's stain counter-stained with light green
- , 2 Eosinophil intra-cellular bodies giving the impression of bursting out of the containing cell to scatter in the surrounding tissues From dog Mann's stain
- „ 3 Eosinophil intra-cellular bodies giving the impression of bursting out of the containing cell to scatter in the surrounding tissues but showing larger eosinophil bodies From dog Mann's stain
- , 4 Eosinophil intra-cellular bodies of navicular form From dog Mann's stain
- „ 5 Eosinophil intra-cellular bodies pressing the nucleus of the containing cell to the periphery From dog Mann's stain
- Figs 6 and 7 Eosinophil intra-cellular bodies of large size From man Mann's stain
- Fig 8 Polymorphous bodies in an excretory duct *Note* the striated epithelium From dog Mann's stain counter-stained with light green
- „ 9 Nerve ganglion in sub-maxillary gland *Note* two Negri bodies in one of the nerve-cells From dog Mann's stain counter-stained with light green
- „ 10 Small magenta-coloured granules in the interacinar supporting tissues of the sub-maxillary gland *Note* the close connection of some of the containing cells to the capillary endothelium and the fact that some of the granules are actually within the latter between the two red cells From dog Mann's stain
- „ 11 Larger magenta-coloured granules in a macrophage From dog Mann's stain
- „ 12 Larger magenta-coloured granules in elongated endothelial cell From dog Mann's stain
- Figs 13 and 14 Inclusions in capillaries From dog Mann's stain

EXPLANATION OF PLATE XXX

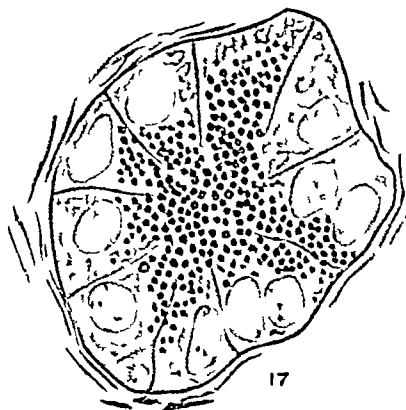
- Fig 15 Dichromic ovoid bodies in cells of serous acini From parotid gland of man
Mann's stain
- „ 16 Serous cell completely occupied with dichromic ovoid bodies of all sizes
From parotid gland of man Mann's stain
- „ 17 Granules in cells of serous acinus *Note* characteristic grouping in central
area with clearer periphery From parotid gland of man Mann's
stain
- „ 18 Dichromic ovoid bodies in serous acinus *Note* well-staining nuclei From
parotid gland of man Iron-hæmatoxylin counter-stained with neutral
red
- „ 19 Eosinophilic vacuole-like structures in crescent of Gianuzzi From dog
Mann's stain
- „ 20 Magnified appearance of eosinophilic vacuole-like structures *Note* internal
vacuoles Mann's stain
- „ 21 Multiple aggregations of irregularly ovoid bodies in serous acinus From
dog Iron-hæmatoxylin counter-stained with neutral red
- „ 22 Eosinophilic vacuole-like structures in a serous acinus From dog Mann's
stain counter-stained with light green
- „ 23 Granules in the serous cells of a mixed acinus *Note* the 'peppering' with
magenta-coloured granules From monkey Mann's stain



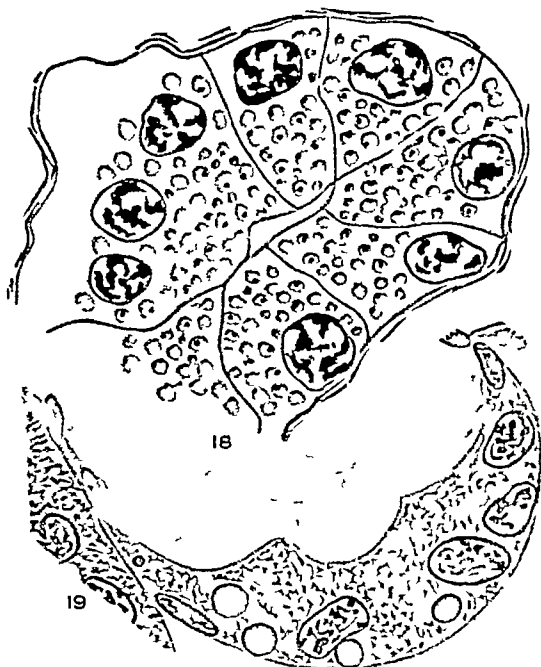
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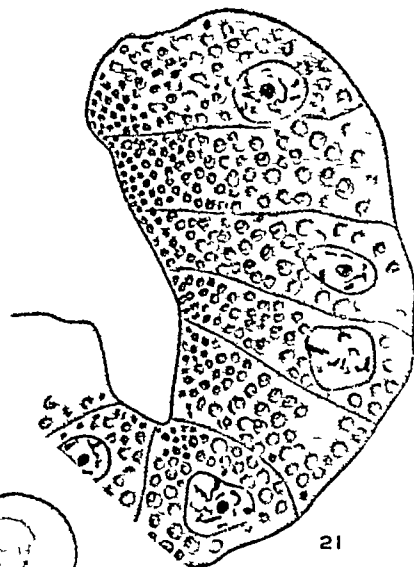
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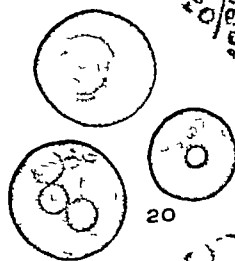
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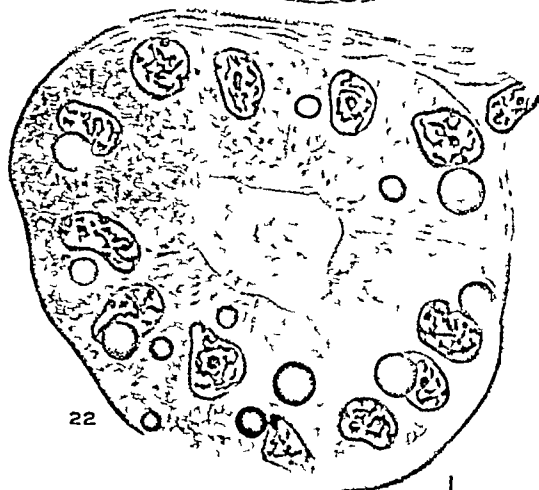
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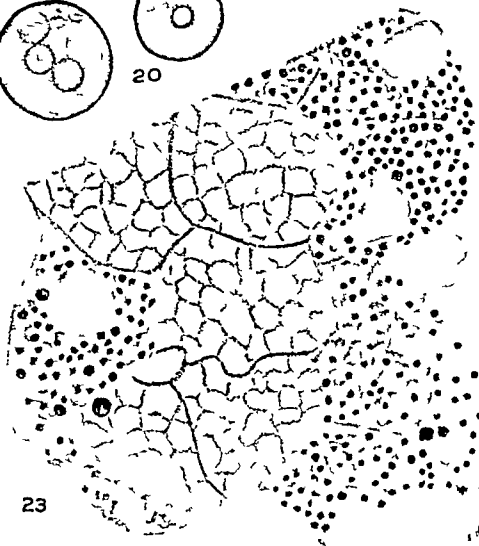
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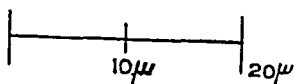
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23



The nuclei of the containing cells, contrary to what we might have expected, showed no evidence of degeneration so far as staining reaction could show it

Iron-haematoxylin —By this staining method the simulation of protozoa was maintained, i.e., the blue matrix stained a light grey colour, easily differentiated away, while the red masses stained a black colour like nuclei. It was found that all groups of the bodies did not differentiate evenly and that while some were stained typically as described above, other groups subjected to the same amount of differentiation had lost their 'nuclei' and only a counter-stain showed up the matrix clearly (Plate XXX, fig 18).

The question now arises as to whether these bodies are homologous with those described in the case of rabid dogs in section IV (c3) as multiple aggregations of irregularly ovoid bodies and, if so, what is their origin

It is unfortunate that the appearances in dog and man which we wish to compare were obtained in the former by the use of Flemming's fixative and in the latter by the use of Sansom-Carnoy fixative

The greater energy and infinitely greater penetrative power of the latter reagent compared with the more slowly acting and poorly penetrating Flemming's solution might be quite sufficient to account for the more sharply cut picture and more distinctive staining obtained in the human cases as compared with the results obtained in dogs. The location, distribution and size of the bodies were approximately the same in man and dog. The fact that they occurred more commonly in man is possibly due to the fact that most of the human cases observed by us lived longer than any of the dogs. The bodies under consideration were only found in dogs in cases which had shown symptoms of rabies for at least two days while some of the human cases survived for five days and one for six days. The bodies were found most plentifully and typically in the human cases surviving the longest time and this might account for their rarer occurrence in dogs which seldom survive three days after the onset of symptoms

We feel inclined therefore provisionally to suggest that the bodies seen in dog and man are homologous structures, any differences being due to unimportant factors necessarily varying with the species of animal

The question as to the nature of the bodies is more difficult to determine and for solution would probably require excursions into the domain of microbiological chemistry which we have not had time to make

We have not, as in the case of some of the other structures previously described, been able to reproduce the bodies under discussion by the action of chemical agents of stimulation of the glands such as pilocarpine so that they cannot be simply explained as secretion coagulated by the fixative. We suggest, however, that they are probably due to the action of an energetic and rapidly penetrating fixative on the cytoplasmic cell contents of serous cells which, due to the presence in them of the rabies virus or its toxin have been so altered either physically or in their secretory function as to respond to the fixative by coagulation or possibly fragmentation of the cytoplasm into the forms described. This view is possibly strengthened by the fact already observed that the bodies were not seen in Flemming-fixed material. It is well known to cytologists that the osmium tetroxide in Flemming's solution

tends to give a comparatively homogeneous fixation of cytoplasm while the mercuric chloride in the Sansom-Carnoy fixative would act as a powerful precipitant of the proteins present in cytoplasm. This explanation is only given as a suggestion and is not meant to rule out the possibility that the bodies may be pre-formed in the cell—they might exist as some precursor of secretion in globules or might, on the other hand, represent complete degeneration of the containing cell the contents of which the fixative coagulated into the form described. Were such the case, however, one would have expected the nucleus also to show some signs of degeneration but this was not the case. One is forced therefore to the conclusion that the bodies are most probably pre-formed globules of the specific zymogen of the serous secretory cells.

In normal white mice injected with pilocarpine nitrate a few serous cells sometimes show an indication of aggregations of large granules. These have neither the sharply cut outlines nor the brilliant staining reaction of the bodies we are describing but are sufficiently suggestive to indicate the possibility that we are dealing with a natural secretory phenomenon exaggerated by hyper-stimulation of the secreting cells by the presence of the rabies virus or its toxin.

2 *Fat vesicles*

As already observed these were sometimes so numerous in the serous cells in human cases as to obscure the histology of the tissues when these were fixed in Flemming's solution.

3 *Other forms met with in human cases*

All the forms mentioned in section (4c) besides those especially dealt with here in connection with human cases were met with in the latter but the large eosinophilic vacuole-like structures [section 4 (c1)] although present were never as numerous as in the dog. The granules in serous acini described in section 4 (b4) tended, when present, to be larger than in the dog and monkey but were probably similar in origin. They tended to show a characteristic grouping in the central area of the acinus the periphery remaining comparatively clear (Plate XXX, fig 17).

DISCUSSION

Manouélian (1914) has described the histological appearances found in salivary glands in rabies. A study of his paper and figures shows little in common with our findings and his explanation of the red-staining granules found in the gland tissues differs from ours. According to him the acidophil granules found in acini are the result of destruction and fragmentation of the nuclei of invading polynuclear leucocytes which became acidophil. As we have shown we were able to produce both the granular and the larger acidophil bodies described by us by the administration of pilocarpine to normal animals. As these results were obtained sometimes within half an hour of the administration of the drug there would certainly have been no time for invasion of the acini by polynuclear leucocytes, their destruction and the conversion of their nuclei into granules.

In a later paper Manouelian and Viala (1924) describe what they consider to be the parasite of rabies as occurring in the cells of the salivary glands. They do not seem to specify which glands they occur in nor whether they occur in serous or mucous cells or in both. They describe these parasites as elongated, fusiform, pyriform, or spindle-shaped and averaging 1μ to 2μ in size. They possess a cytoplasmic body staining a bright red with Mann's stain surrounded by a membrane and containing granules of chromatin which may be rounded or filamentous and stain blue. These bodies are said to occur both in parts of the glands little altered from the normal and in parts showing considerable lesions. They are found in the cells of the acini, sometimes widely disseminated, sometimes aggregated in a small vacuole. They are also seen sometimes in large numbers in the salivary ducts and hence must reach the saliva. As a perusal of the description of our own findings will show we met nothing corresponding with these parasites in our series of cases from dog, monkey and man.

A consideration of our description will further bring to light another fact, viz., that of all the various conditions described by us as occurring in the salivary glands in rabies it is at least doubtful whether any can be considered as exhibiting the occurrence of 'inclusion bodies' such as one might expect in a virus disease, if rabies is so. A possible exception is the case of the bodies described in section 4 (b2). These appear to us to be very similar to bodies described in the case of infectious ectromelia by Marchal (1930) in connective tissue cells.

A study of Marchal's figure of a salivary gland stained by Mann's stain was also very suggestive of some of our findings. The red bodies in his description, however, are considered to be genuine inclusion bodies while we have explained the bodies we found as coagulated secretion or its precursor and have actually produced an identical condition by the use of pilocarpine. A third figure of Marchal shows inset an enlarged representation of one of the inclusion bodies seen in the cells of the pancreas. This appears to be identical in structure with the bodies described by us in section 4 (c1) as a study of the two figures will show. Now our bodies are definitely secretory in nature and were reproduced by us in dogs by the use of pilocarpine. As the pancreas is also a secretory gland it seems a possibility to us that there also the so-called inclusion bodies may be due to the action of the fixative on a gland in a condition of hyper-secretion.

CONCLUSIONS

1 A prolonged study of a large amount of rabid material, including dog, monkey and man, has revealed that while characteristic appearances are found in the salivary glands of rabid animals, these are in most cases the results of an exaggerated physiological response of the glands to hyper-stimulation and do not necessitate the specific presence of a living virus, although this is probably the cause of the stimulation in rabies.

2 No evidence was obtained of the visible presence of any bodies which could be identified as bacteria, protozoa, or filterable viruses, or indeed, of any living causative agent.

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THE PHARMACOLOGICAL ACTION OF THEVETOXIN
A SECOND GLUCOSIDE FROM *THEVETIA*
NERIIFOLIA

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CHOPRA and MUKERJEE (1933) have recently published an account of the chemistry and pharmacological action of Thevetin, a glucoside isolated from *Thevetia nerifolia*. This glucoside was first isolated by de Vrij (1881, 1882, 1884) from the kernel of the seeds, but as he did not mention its formula or the melting point, it is probable that he was not able to obtain the glucoside in a pure form. De and Chowdhuri (1919) succeeded in obtaining Thevetin in a pure form with a melting point of 189°C to 190°C and gave its formula as $C_{72}H_{124}O_{36}$. They also obtained from the non-sugar product (of hydrolysis of the glucoside) an amorphous substance stated to be more toxic than Thevetin and named it Thevetidin. The presence of a second poisonous principle was also indicated earlier by Warden (1882).

Ghatak (1932), working at the Chemical Laboratory of the Allahabad University under Dr Dutt, isolated the second poisonous principle in a pure glucosidal form and, on account of its highly poisonous character, named it 'Thevetoxin'. He used the following method of isolation. The oil-free kernels were completely freed from petroleum ether and successively extracted with rectified spirit, till a portion of the extract gave only traces of residue on complete evaporation. The alcoholic extract was concentrated under reduced pressure, when a thick-brown syrupy liquid, strongly smelling of sugar, was obtained. This slowly solidified to a brown hygroscopic mass in a vacuum desiccator, on extraction with chloroform it gave 40 g of yellowish brown solid after complete evaporation of the solvent. This substance was completely soluble in ethyl acetate. Traces of oil that contaminated it were

removed by treatment with petroleum ether. On crystallization from dilute alcohol it was obtained as snow-white slender needles melting at 192°C and having a molecular formula $\text{C}_{20}\text{H}_{30}\text{O}_6$. This was probably the substance which was isolated in the crude form by de Vrij and named by him as Thevetin. This substance is insoluble in water but easily soluble in most of the organic solvents. It does not reduce Fehling's solution or Tollen's reagent but both of them are readily reduced if the compound is previously hydrolysed by warming with concentrated hydrochloric acid. It does not produce any coloration or precipitate with the usual alkaloid reagents. Alcoholic ferric chloride, lead acetate or sub-acetate, silver nitrate or calcium chloride have no effect on the substance. When perfectly pure the substance is quite tasteless, but in the presence of only traces of impurities it has a pronounced bitter taste. The substance has been named as Thevetin by the present author, after de Vrij who was the first worker in the field.

The residue left after chloroform extraction was freed from the solvent and dissolved in 400 c.c. of water. The liquid which was deep brown in colour was allowed to stand with few drops of chloroform to arrest bacterial growth. After about a week a white sediment started separating from the mother liquor and within a fortnight the whole of the vessel was full of a white shining deposit. The product was filtered at the pump and on drying weighed 37 grammes. This, on twice re-crystallization from hot water, was obtained as slender shining silky needles melting at 178°C . This substance which has the molecular formula $\text{C}_{16}\text{H}_{24}\text{O}_8$ differs from Thevetin by its solubility in water and intense bitter taste. In its chemical deportment, however, it resembles Thevetin very closely, giving no reaction with Fehling's, Tollen's and alkaloid reagents and no precipitate with silver nitrate, lead acetate, ferric chloride or calcium chloride. Basic lead acetate, however, produces an abundant flocculent white precipitate on warming. This substance on hydrolysis reduces Fehling's solution and Tollen's reagent readily. It dissolves in strong sulphuric acid, with orange coloration, which intensifies in about five minutes and becomes deep red. It is insoluble in most of the organic solvents except alcohol in which it is very soluble. It is sparingly soluble in acetone and moderately soluble in water from which it crystallizes in slender needles. It is optically active having a *lævo*-rotation of $[\alpha]_{\text{D}}^{30} = -76.1$ in ethyl alcohol.

We feel greatly indebted to Dr. Dutt for supplying us this glucoside which enabled us to work out its pharmacological action.

PHARMACOLOGY

Procedure—All experiments were carried out on healthy dogs, cats, rabbits and frogs. In toxicity experiments, guinea-pigs were also used. In dogs morphine-urethane anaesthesia was used in doses of 5 mg. of morphine and 1.8 g. of urethane per kilogram of body-weight. In cats decerebrate or spinal preparations were mainly used. Fresh solutions of Thevetoxin 1 mg. per c.c. in 0.9 per cent saline were chiefly used.

All injections were made at body temperature.

External actions—

(a) *LOCAL ACTION*—In concentrations up to 1 in 1,000 the glucoside has no irritant action on skin, mucous membrane or subcutaneous tissues. The taste is

bitter Large doses given to cats by mouth did not produce any inflammation of the gastric mucosa

(b) TOXICITY—This was tested on various organisms, particularly with a view to obtain figures which could be compared with those obtained by Chopra and Mukerjee for Thevetin The result obtained is given in a tabular form below —

Thevetin	Thevetoxin
1 <i>Paramacium</i> and <i>helminths</i> —No toxic effect in 1 in 100 000 dilution	No toxic effect in 1 in 200 000 to 1 in 5,000 concentrations
2 <i>Frogs</i> —0.15 mg to 0.3 mg per kilo M L D	25.0 mg to 30.0 mg per kilo M L D
3 <i>Guinea pigs</i> —0.015 mg to 0.024 mg per kilo body weight subcutaneous lethal dose	10.0 mg per kilo body weight S L D
4 <i>Cats</i> —Oral 0.14 g per kilo	Oral 0.25 g proved non fatal Subcutaneously 1.0 mg per kilo as average lethal dose Dogs—1.0 mg per kilo subcutaneously average lethal dose

A glance at the above table reveals the fact that Thevetoxin of Ghatak is much less toxic than Thevetin of Chopra and Mukerjee, whereas the former claimed that the Glucoside Thevetoxin isolated by him was more toxic than Thevetin. The train of symptoms observed with toxic doses in frogs and guinea-pigs were very similar to those observed by Chopra and Mukerjee. In dogs, drowsiness, lethargy and prostration were a marked feature and were, in our opinion, due to depression of the higher centres rather than to mere exhaustion resulting from excessive vomiting and diarrhoea. A dog which was very irritable and noisy before the experiment became quiet within half an hour of the injection. Vomiting, in our opinion, is of central origin as shown later by actual experiments. It appeared in half an hour to one hour after subcutaneous doses but with oral doses it took 2 to 3 hours and needed 10 to 20 times bigger doses. The only noticeable result of a previous dose of atropine was decrease in frequency of vomiting and salivation, showing that vagal inhibition played very little part in producing death from cardiac failure.

Circulatory system—Our experiments confirm what earlier workers have shown that the main action of the drug is exerted through the circulatory system. As early as 1876 Blas and Hausmann stated that Thevetin had on frogs an action similar to that of digitalis. Chopra and Mukerjee (1933) also found that Thevetin had a pronounced effect on the circulatory system which resembled in many respects the action of drugs of the digitalis group. Thevetoxin closely resembles Thevetin in its cardiovascular action. Few points of difference between Thevetin and Thevetoxin were, however, observed. We give these in detail below.

Injection of 0.1 mg to 0.5 mg of Thevetoxin into the femoral vein of an average size cat (2 to 3 kilo) caused within a few seconds a marked rise of blood-pressure, varying from 30 mm to 80 mm of mercury. This was not well sustained and in 5 to 10 minutes came to normal (Graph 3, figs *f* and *g*). In dogs the rise in pressure was less marked but it was more sustained (Graph 3, figs *a*, *b* and *c*). A higher degree of pressor effect was obtained with similar doses in spinal cats which showed that the vasomotor centre played no part in the rise of blood-pressure and further that the stimulation of the vagal centre, in the intact animal to some extent neutralized the pressor effect of the drug (Graph 3, figs *f* and *g*). The pressor action did not disappear or lessen when sympathetic ganglia were paralysed with nicotine. Injections of ergotoxin acid phosphate which were capable of producing reversal of adrenaline effect did not in any way alter the pressor action of Thevetoxin. Cocaine in doses sufficient to sensitize the sympathetic receptive substance to adrenaline (15 mg per kilo) did not increase the magnitude of the pressor effect. These observations definitely showed that the sympathetic mechanism played no part in the pressor action of this glucoside.

Perfusion of hind legs of the frog (Loewen-Trendelenburg method) with 1 in 100,000 solution of Thevetoxin did not produce any appreciable effect. The rate of outflow changed from 4.5 to 4.4 c.c. per minute. Perfusion of dogs' and cats' hind legs through the femoral artery were also without any appreciable effect. Perfusion of the hind legs of the dog was next performed through the abdominal aorta, using a perfusing pump, and recording the pressure with a mercury manometer, connected to the side tube of the arterial cannula. The pump worked always at about 82 strokes per minute and throughout an experiment the output per stroke was kept constant. The tracing in Graph 3, fig *h* shows the effect of injecting 0.25 mg of Thevetoxin when the limbs were being perfused with 0.9 per cent saline solution of the drug at 37°C. There is hardly any effect in the peripheral resistance—tracing in Fig *2* shows the effect of the same dose, when the perfusing solution was changed to freshly-defibrinated blood. The peripheral resistance now rose slightly. Adrenaline (1 in 20,000) was then added drop by drop to the reservoir into which the venous output from the legs emptied. As the blood containing adrenaline circulated through the legs peripheral resistance rose gradually till it maintained a steady level, tracing in Fig *7* shows the effect of same dose of Thevetoxin at this stage. The rise in peripheral resistance was greater now than with defibrinated blood alone. This showed that for the constrictor effect of Thevetoxin presence of adrenaline was necessary. We were familiar with Burn's work on Tyramine and Ephedrine, where in it was shown that adrenaline augmented the constrictor effect of Tyramine and Ephedrine. We repeated the above experiment by increasing the tone with post-pituitrin extract. Similar results were obtained to those obtained with adrenaline and we conclude that for the constrictor effect of this glucoside initial tone in blood vessels, as is maintained during health by continuous liberation of adrenaline, pituitrin, etc., is necessary. The constrictor effect noticed in the perfusion experiments was much less compared to the pressor effect obtained in intact animals and from this we conclude that part of the pressor effect is due to stimulation of the heart. This was further borne out by the fact that intestinal, renal and spleen volumes showed slight increase during the pressor effect of the glucoside.

Action on the heart —Frog's heart Graph 1, figs *d*, *e*, *f* and *g* illustrate the action of this glucoside on frog's heart. Tracing in Fig *d* shows the effect of 0.1 mg, injected intra-hepatically in a decerebrate frog, the immediate effect was the absence of complete ventricular relaxation within a few seconds the height of systolic contractions increased and diastolic relaxation came back to its original. No slowing was observed and within a few minutes the heart came back to normal. Tracings in Figs *e*, *f* and *g* show the effect of a bigger dose, 0.25 mg, here again stimulation of auricles and ventricles is noticed, followed by slowing of auricles from 52 to 40, and irregularity of ventricles due to partial heart block, half an hour later the auricular rate had become 12 per minute and ventricle was receiving regular impulses from auricle—the force of contraction of both the auricle and ventricle was greatly increased. Every now and then a few beats at double the rhythm alternated in between as if the conduction was recovering temporarily. This phase continued for a long time and injection of 2 mg of atropine made only a slight difference to the rate, Fig *g*, but increased the force of contraction. With bigger doses the same events were noticed to follow at quicker succession and ultimately the heart stopped, sometimes in systole and at others in diastole.

Mammalian heart Graph 1, figs *a*, *b* and *c* illustrate the effect of Thevetoxin on isolated rabbit's heart, perfused through the aorta, tracing in Fig *a* shows marked stimulation which followed an injection of 0.25 mg, tracing in Fig *b* shows the events half an hour later when the heart rate had slowed down to half but there was no irregularity and the original stimulant effect was passing off. Another injection of 0.25 mg again produced some stimulation accompanied with regular extra systole after each beat, a few minutes later the heart became quite irregular and finally stopped in diastole (Fig *c*). Graph 1, figs *h*, *i* and *j* show a myocardiogram from a decerebrate cat which had two injections of 0.25 mg and 0.5 mg. It depicts some of the various types of irregularities which have been met with during our work on this drug. Tracing in Fig *h* shows the periodic variation in the systolic and diastolic excursions which take place when auricles and ventricle beat at a slightly different rate and which has been described during the toxic phases of digitalis and strophanthin (Cushny, 1925). The ventricle also shows a few extra systoles. In the first part of the tracing in Fig *i* both the auricle and ventricle were beating irregularly. At X the drum was stopped for 10 minutes when both the auricles and the ventricles were found to have regained the normal rhythm, the vagi were now cut and another 0.5 mg was injected, marked irregularity of the auricles and ventricles again made its appearance. After few minutes both the auricles and ventricles began to fail and there were long periods without any cardiac cycle and then a few ventricular and auricular beats escaped through at irregular intervals, before the heart completely stopped with ventricles in full relaxation and auricles well contracted. Cardiometer experiments with decerebrate and spinal cats also showed stimulation and then marked increase in the heart volume and finally arrest in full diastole (Graph 3, figs *f* and *g*).

Further investigations into the nature of the cardiac irregularity were made with the electrocardiograph on anaesthetized dogs. This was made possible by the kind help and co-operation of the Physics Department of the Lucknow University and we wish to express our grateful thanks to Dr Wali Mohammad and Dr K N Mathur. Graph 2, fig *a* represents the normal electrocardiograph of a dog of 4 kilo body-weight under urethane-morphine anaesthesia, Fig *b* represents the effect

of 0.25 mg.—there is no change in the electrocardiograph, Fig *c* represents events after the dog had received 2 mg of the glucoside. In lead 1 the auricular beats are coming at irregular intervals and the P-R interval at 1 and 3 is bigger than normal. This shows that there is partial block between the node and auricles and as well as between the auricles and ventricles. In leads 2 and 3, which were taken a few minutes later than lead 1, it appears that there is idioventricular rhythm from the left ventricle and no impulses are arriving from the pace-maker. Fig *d* which was taken after 4 mg of atropine shows return of P waves marked P, but P-R is very long and of varying length. There also appears to be left branch block. After another 1.5 mg the dog expired suddenly and we were unable to take electrocardiographic record of terminal events.

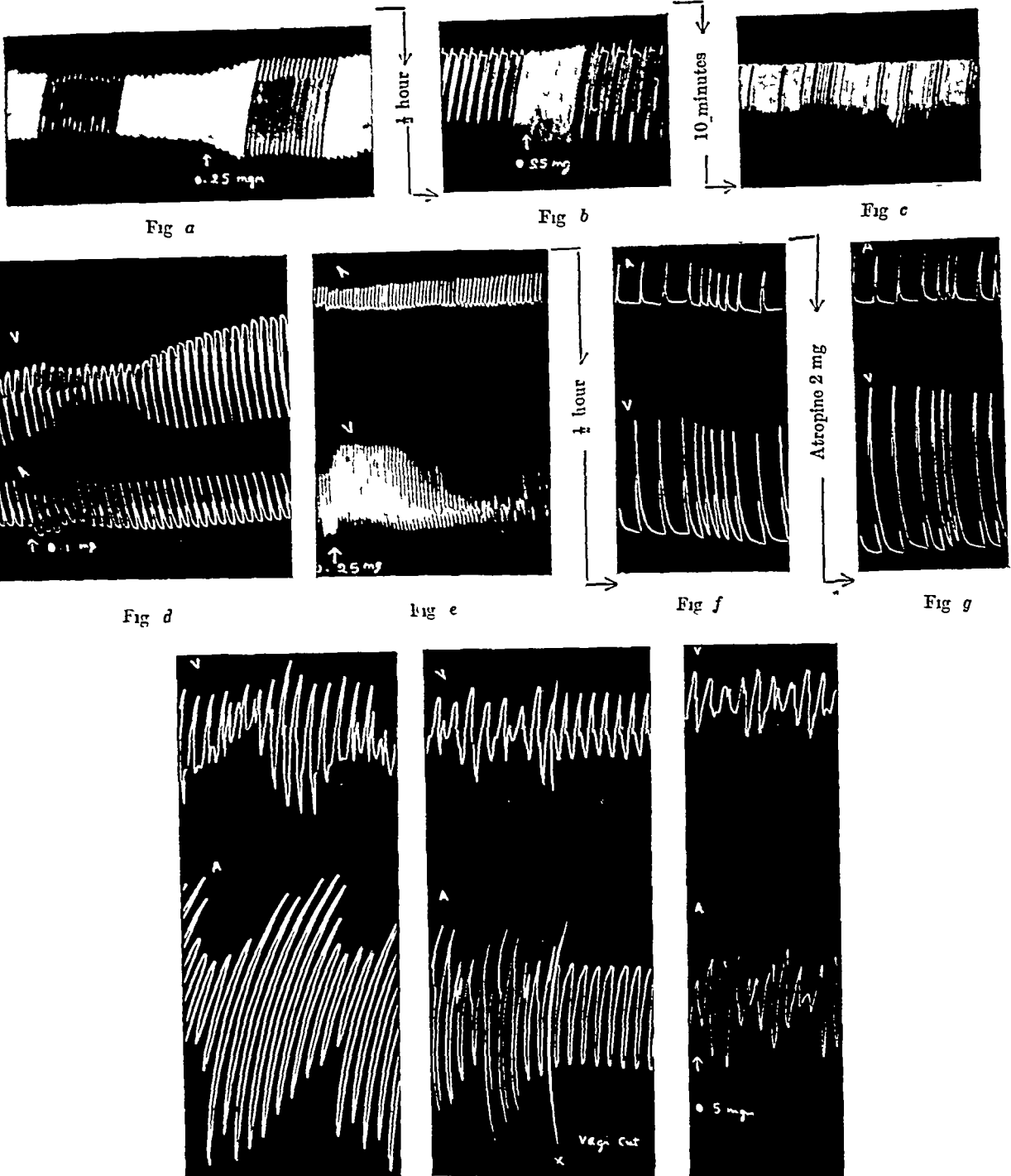
Alimentary system—The normal rhythmical movements of the ileum of the dog *in situ* (finger-cot method) were first stimulated and then the movements decreased but there was gradual increase in tone (Graph 3, fig *b*). This action was present though less marked after severance of vagi and atropinization of their nerve-endings. The excised strips of ileum of the cat show an increase in the tone and the amplitude of contractions (Graph 3, fig *d*). Atropinization of the vagal nerve-endings was effective in bringing down the tone, so probably the effect was both through the stimulation of the vagi and a direct action on the plain muscles.

Respiratory system—In all the animals in which toxicity experiments were conducted, appreciable changes in the rate and character of respiration were noticeable. The rate usually became slow though sometimes, and particularly in the initial stages, was quickened. There was invariably an increase in force and the respiratory excursions assumed a laboured character. The accessory muscles of respiration were often seen to come into action. Graph 3, fig *a* shows the record of tracheal pressure with a Marey's tambour from a dog under morphine-urethane, with 0.5 mg there is a marked increase in the pressure, as the vagal nerve-endings were paralysed so the action seems to be a direct one.

Uterus—*In situ* as well as in excised strips increase in tone was observed (Graph 3, figs *c* and *e*).

Nervous system—General depression and drowsiness was a marked feature observed in animals on which toxicity experiments were carried out. This came on quite early with a big dose but with smaller and non-fatal doses was usually preceded by a period of irritability, excitement and tremors. Convulsions were noticed near death particularly in guinea-pigs. It is said that prostration and drowsiness are due to a state of collapse resulting from excessive vomiting and purging, but we noticed drowsiness and paresis even in animals in which no vomiting and purging takes place (guinea-pigs and rabbits), and in dogs it sometimes precedes vomiting. This leads us to believe that the drug has a depressant effect on the higher centres and stimulant effect on the lower (medulla and cord) which accounts for the stimulant action of the respiratory and vagal centres and also for tremors and twitchings. Convulsions which were observed only near death were probably due to anaemia of the brain resulting from failing heart. Vomiting, in our opinion, is also a central effect, for this was observed much earlier and with smaller doses when the drug was administered subcutaneously than when given by mouth. Besides, on post-mortem examination of cats which had received large doses by mouth no signs of irritant effect on the stomach-wall or intestines were observed.

GRAPH 1



Figs a b and c—Rabbit's isolated heart, perfused with Locke's solution (Legendari's method) Upstroke=svstole Figs d, e, f and g—Decerebrate frog A=Auricle, V=Ventricle, Upstroke=svstole Figs h, i and j—Myocardiograms from a decerebrate cat (2.2 kilo) Ventricle (upper) at Fig h, the cat had 0.75 mg of Theobromine and is showing the toxic phase of the drug, each beats in regular rhythm, but at slightly different rates and this irregularity gives rise to periodic variation in the systolic and diastolic excursions

GRAPH 2

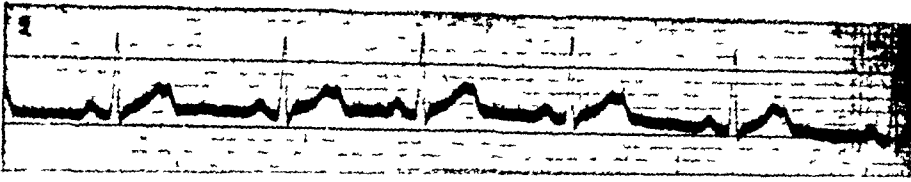


Fig a



Fig b



Fig c

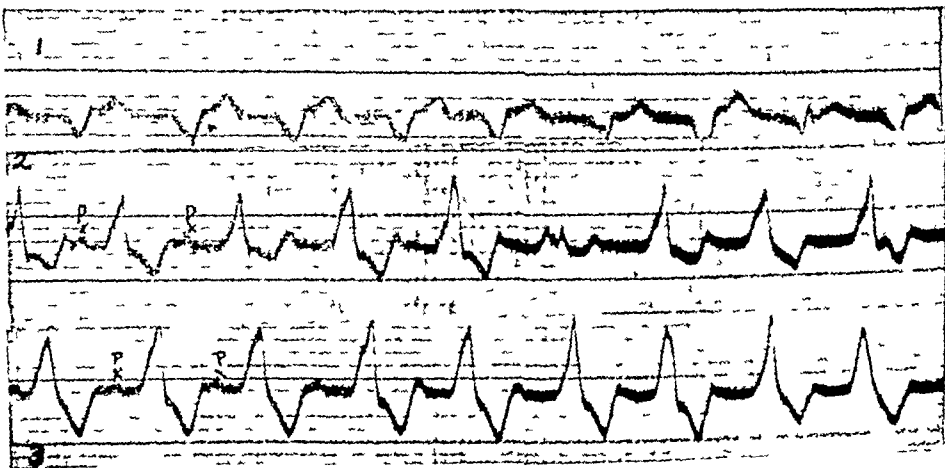


Fig d

Fig a—Electrocardiographic tracing from lead 2 of a dog (4 kilo) under morphine-urethane anesthesia, sinus arrhythmia is apparent. Fig b—Tracings of lead 2 of the same dog, after it had 0.25 mg of Thevetoxin—no changes are evident. Fig c—leads 1, 2 and 3, from the same dog, after it had a total of 2 mg of Thevetoxin. In lead 1 the beats are coming at irregular intervals and at 1 and 3 P-R interval is longer. In leads 2 and 3, which were taken a few minutes later, idioventricular rhythm seems to be present. Fig d—The dog had now 4 mg of atropine—it appears as if left branch block has set in.

GRAPH 3



Fig a

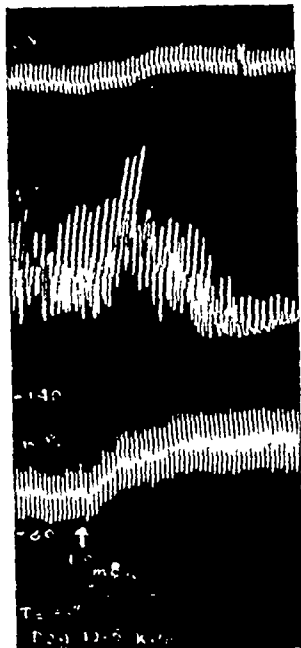


Fig b



Fig c

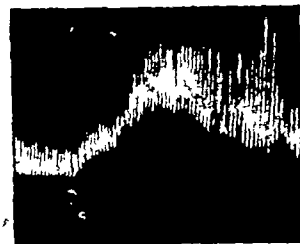


Fig d



Fig e



Fig f

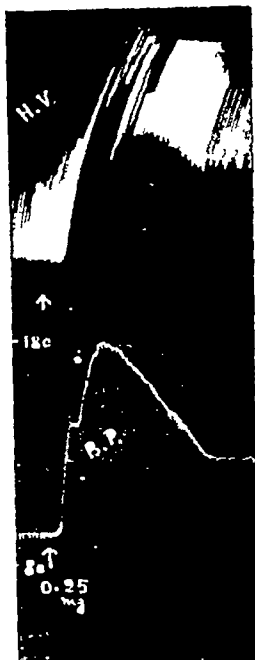


Fig g



Fig h



Fig i



Fig j

Fig a—Dog (7 kilo), vagi cut, vagal ends atropinized. The tracing shows changes in blood pressure and tracheal respiration (records with Marey's tambour) after an injection of 0.5 mg of Thevetoxin. Fig b—Dog (10 kilo). The tracing shows changes in intestinal movements (finger cot method) and blood pressure with 1 mg of Thevetoxin. Fig c—Dog (12.5 kilo). The tracing shows changes in uterine horn (upper) and blood pressure with 1 mg of Thevetoxin. Fig d—Isolated ileum of cat suspended in 150 cc of Tyrode's solution at 37 C. Fig e—Isolated uterus of cat suspended in 150 cc of Tyrode's solution. Fig f—Decerebrate cat (2.2 kilo). The tracing represents changes in heart volume (H. V.) and blood pressure with 0.25 mg of Thevetoxin. Fig g—Shows the above events in a spinal cat (2.5 kilo). Figs h, i and j—Records of pressure in the arterial cannula secured in the abdominal aorta of a dog, just above the bifurcation to hind limbs, Fig h, when saline was used for perfusion, Fig i, when it was changed to freshly defibrinated blood and Fig j, when adrenaline was added to the reservoir. Dixon's pump was used for perfusion.

DISCUSSION

It would appear that the most important action of Thevetoxin like that of Thevetin is on the cardiovascular system. The nature of this action varies with the doses employed. Smaller doses stimulate the systolic contractions of the heart and produce marked rise in blood-pressure. Toxic doses tend to produce cardiac irregularities as in the case of the digitalis group of drugs. That this action is in part due to the stimulation of medullary centres is proved by the fact that changes in blood-pressure and heart rate were affected differently in decerebrate, spinal and intact cats. That the drug also acts on the peripheral vagal mechanism is shown by the fact that the conduction disturbances, particularly those occurring between the pace-maker and the auricle, were due to the stimulation of vagal nerve-endings. But the fact that marked cardiac irregularities were noticed both in frogs as well as in cats and dogs, after the vagal nerve-endings were efficiently paralysed, makes us believe that though the drug acts to a certain extent through the medullary centres and the vagal nerve-endings, the major action of this glucoside is on the muscle fibres of the heart. With small doses contractile fibres are stimulated but with toxic doses it is the conductile system which is most adversely affected, which accounts for the fact that cardiac arrest nearly always takes place in diastole. Thevetoxin thus seems to differ from the digitalis group of drugs in having a more potent action on conductile fibres and a feebler action on the contractile tissues. In its toxic action the whole of the conductile system of the heart seems to be affected, for the slowing of the heart as a whole as in Graph 1, fig *b*, and long pauses during which no cardiac cycle seems to take place, are evidences of the fact that either the pace-maker is affected or there is supra-auricular block. The prolongation of P R interval (Graph 2, figs *c* and *c'*) and occasional failure of the impulse to reach the ventricles points to a block between auricles and ventricles. Lastly, the broadening of Q R S complex and its notching shows that conduction through the terminal branches of the bundle of His is also affected. Ultimate failure of the heart is sometimes preceded by ventricular fibrillation and sometimes the pace-maker seems to fail.

The pressor effect of the drug which is well marked is partly through the constriction of the blood vessels and partly through the stimulation of the heart. For the constriction effect presence of initial tone in the blood vessels seems to be necessary, for no such effect was noticed when blood vessels of frogs, cats and dogs were perfused with Ringer's solution or defibrinated blood, but when pituitrin or adrenaline was added to the perfusing solution, the pressor effect was well marked. However, the extent of pressor effect which was obtained in perfusing experiments was much less compared to that obtained in intact animals, this fact along with the observations that the spleen, kidney and intestinal volumes increased during rise in blood-pressure makes us believe that major part of the pressor effect was through stimulation of the heart.

The glucoside has a similar stimulant action on the plain muscles of the intestine, uterus and bronchi.

Thus, in their general pharmacological action the two glucosides of *Thevetia nerifolia* resemble each other closely, the main difference being in their toxicity. Of the two, Thevetoxin appears to be much less toxic and has no claims to the name given to it by Ghatak.

Thevetoxin also closely resembles drugs of the digitalis group in its action on the cardiovascular system with this difference that action on the conductile system of the heart is more marked than on the contractile tissues. Thevetoxin has no local irritant or anæsthetic action like digitalis and strophanthus.

SUMMARY

(1) Thevetoxin is a second glucoside from *Thevetia nerifolia* Ghatak, working at the Chemical Laboratory of the Allahabad University, isolated it, by a method which is described above.

(2) Thevetoxin closely resembles Thevetin of Chopra and Mukerjee in its general pharmacological action but differs in being less toxic. The name of Thevetoxin given by Ghatak appears unsuitable as it implies greater toxicity.

(3) Thevetoxin has a stimulant action on the plain muscles of the intestine, blood vessels, bronchi, uterus and heart.

(4) The cardiovascular action of Thevetoxin closely resembles that of the drugs of the digitalis group with this difference that the action on the conductile system is more marked. This accounts for the cardiac arrest in diastole.

(5) Unlike digitalis and strophanthus, Thevetoxin has no local irritant action.

(6) Thevetoxin, though less toxic than Thevetin, is yet toxic enough to prevent its safe use as a therapeutic agent.

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BLOOD-GROUP DISTRIBUTION IN THE TODAS

BY

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THE Todas are a primitive people living on the plateau of the Nilgiris, the hill resort of Southern India. In spite of their small number (it was 597 at the Census of 1931) they have attracted a good deal of the attention of the ordinary visitor and the anthropologist, owing to their quaint and unique customs. They are a pastoral people whose only occupation is the tending of buffaloes. Even their religion is mixed up with these creatures to a large extent. They are the earliest settlers of the district and are considered as 'lords of the soil'. Of their social customs brief mention may be made of polyandry, polygamy and female infanticide. This last practice has died out in recent years. Polygamy has perhaps crept in recently. Both polyandry and polygamy are of the fraternal type where one or more wives are shared by brothers. The Todas live in complete seclusion and there has been no instance of inter-marriage with other communities.

The Todas are so different from the other inhabitants of the region that speculation has been rife as to their origin. Various theories built upon insufficient evidence need not be mentioned here. Dr Caldwell, quoted by Grigg (1880), thinks that they are a Dravidian race who left the plains after the Aryan invasions, but before the tenets of Brahmanism had taken any hold upon the minds of the people and before there had been any extensive mixture of races. Rivers (1906) who made a thorough study of the Todas is inclined to the view that they are of Dravidian origin and came from the direction of Malabar. He is not certain of the time of migration, but it must have been some centuries ago.

When an investigation was being conducted by the King Institute (Pandit, 1927) into the health of the Todas, the opportunity was taken to determine the distribution of blood-groups among them. A census which was conducted at the time showed that there were 582 Todas, and bloods of 200 individuals were grouped. The two test-sera were obtained from Lieut-Colonel R B Lloyd, I M S, Imperial Serologist, Calcutta, to whom our thanks are due. The microscopic test was employed.

TECHNIQUE

Two drops of blood were received into 5 c c of citrated saline. This was the cell suspension. A drop of groups A and B sera were placed separately on a slide and each drop was thoroughly mixed with a drop of the cell suspension. After a

short interval, a cover-slip was placed on each mixture, and after standing at room temperature for 15 to 20 minutes, the preparation was examined under the low power of the microscope for presence or absence of agglutination. If the cells are agglutinated by group-A serum alone, the blood belongs to group B, if by group-B serum alone, to group A; if by both sera to group AB and if by neither, to group O.

The distribution of the groups and the values of the frequencies p , q and r arrived at, are given in Table I —

TABLE I

O	A	B	AB	TOTAL
59 (29.5 per cent)	39 (19.5 per cent)	76 (38.0 per cent)	26 (13.0 per cent)	200

$$p = 17.8, q = 30.0, r = 54.3 \text{ (percentage)}$$

The values p , q and r representing respectively the percentage frequencies of A, B and O were calculated by using the following formulæ of Snyder (1929) in accordance with the triple allelomorph hypothesis of Bernstein —

$$p = 1 - \sqrt{O + B}$$

$$q = 1 - \sqrt{O + A}$$

$$r = \sqrt{O}$$

The Todas being a primitive Dravidian people of Southern India, who in their seclusion have undergone little or no admixture with other races, it should be interesting to compare their blood-group distribution with those of Dravidian people in other parts of India. Few results of blood-grouping in India have been published*.

Malone and Lahiri (1928) examined, besides other Indian types, the blood of 589 Dravidians consisting of Uraons, Mundas and Santals—aboriginal peoples of Chota-Nagpur. Their results are tabulated below along with those for Todas —

TABLE II

	Number examined	O	A	B	AB	p	q	r
Dravidian type of Malone and Lahiri	589	24.3	27.5	36.8	11.4	21.9	28.1	49.2
Todas	200	29.5	19.5	38.0	13.0	17.8	30.0	54.3

(The figures are in percentages.)

*HIRSCHFELD and HIRSCHFELD (1919) examined bloods of 1,000 Indian soldiers at the Macedonian Front during the Great War. They were, however, dealing with a heterogeneous population consisting of several Indian races.

CHAUDHURI (1931) grouped bloods of 154 Kayasthas from different parts of West Bengal.

It will be noticed that, in the Todas, the frequency of B (*q*) is a little higher and that of A (*p*) is very much lower than in the Dravidian type of Malone and Lahiri

The frequency of A is also lower in the Todas than in the other Indian types or races examined by these authors, namely, Turko-Iranian, Indo-Aryan and Hindus of the United Provinces, in whom the frequency was 18.1, 18.8 and 17.9 respectively. The frequency of B in these races—21.4, 24.1 and 26.1 respectively—is very much lower than in the Todas

SUMMARY

The results of blood-grouping in 200 Todas, a primitive Dravidian people of Southern India, are recorded

These are compared with the frequency figures published for other Dravidian and other races in India by Malone and Lahiri (1928)

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SOME OBSERVATIONS ON THE CARDIOVASCULAR ACTION OF UREA-STIBAMINE

BY

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WHILE the clinical effects produced by urea-stibamine and other allied antimonial salts have been the subject-matter of a great number of papers, the pharmacological action of these compounds has received but scant attention. Chopra (1927), as the results of experiments on cats, stated that the organic compounds of antimony produce a fall of systemic blood-pressure when given intravenously, the effect being due partly to the lessened output of the heart and partly to dilatation of the splanchnic vessels. The pulmonary pressure and that in the inferior vena cava was found to be raised.

The present work was undertaken to extend Chopra's observations. But we used only dogs for all our experiments since these were readily obtained from the municipal lethal chamber and cost practically nothing. The general anaesthesia used was either paraldehyde by stomach tube or morphia subcutaneously followed by chloretone intraperitoneally. The urea-stibamine used was that manufactured by Brahmachari. A small supply was first obtained from him, but later we bought the drug as required from the open market. The results obtained by us are given below —

Action on the heart — The action of urea-stibamine on the dog's heart was studied by means of the myocardiogram in the intact animal. Graph 2, figs a and b, shows the myocardiograph records. Injections of 200 mg to

250 mg. produced a depression of both the auricle and the ventricle, which tended to pass away quickly. In both cases illustrated the effect had disappeared in about $1\frac{1}{2}$ minutes. It will be noticed that in Fig *b* the weakening and dilatation of the cardiac muscle is greatest with the secondary rise of blood-pressure. When continuous intravenous injections were given, up to a maximum of from 600 mg to 1,000 mg, the cardiac contraction became more feeble and the blood-pressure fell markedly and did not return to normal. Taking the blood volume to represent $1/13$ th of the body-weight, the concentration in the blood of a dog weighing about 6 kilos, after an intravenous injection of 200 mg, will be approximately about 1 in 2,000. The ordinary therapeutic dose in human beings is about 200 mg intravenously. A larger amount is rarely given in a single dose. This represents a concentration of about 1 in 20,000 in the circulating blood for a person weighing about 60 kilos. Therefore, the concentration of urea-stibamine in the blood of a dog injected with 200 mg to 250 mg intravenously will show approximately 10 times that obtained in the human blood. Such a big concentration has in the cases illustrated (Graph 2, figs *a* and *b*) only produced a transient depression of the heart. Perfusion of the isolated kitten's heart with as high a concentration as 1 in 2,000 shows a slight but persistent depression of the amplitude of contraction. A 1 in 500 dilution causes only a temporary depression of the perfused isolated heart of the frog (Graph 2, figs *c* and *d*).

Electrocardiograms were taken of the dog's heart before and after an intravenous injection of 200 mg urea-stibamine. Two records were taken, one immediately after injection and another 35 seconds after the injection was given, the duration of both being 28 seconds. In neither case was any abnormality noticed as regards the electric conductivity of the heart or its rate or regularity. Electrocardiograms were also taken of two cases of kala-azar undergoing treatment in the General Hospital, both before and after intravenous injections of 200 mg urea-stibamine. No changes were observable. Studied according to Waddell's technique urea-stibamine was found to have no effect on the refractory period of the turtle's heart.

On blood-pressure — Intravenous injections of about 200 mg to dogs weighing about 5 to 6 kilos produced somewhat varying effects. In most cases, immediately following the injection there was a slight fall of blood-pressure and this was succeeded by a rise which was very marked in some cases, *vide* Graph 1, fig *a*, Graph 2, fig *b*, and Graph 3, fig *a*. In a lesser number of cases there was little change in the blood-pressure after injection, e.g., Graph 3, fig *c*. In a few cases only a transient fall of blood-pressure was noticed, *vide* Graph 1, fig *c*, where as much as a total of 500 mg had been administered, also Graph 2, fig *a*, and Graph 3, fig *b*. In cases where the subsequent rise was quite marked it persisted even after doses of ergotoxin which reversed the pressor action of adrenaline, *vide* Graph 1, fig *b*.

In order to find out whether the ordinary therapeutic intravenous dosage of 50 mg to 200 mg in human beings had any effect on blood-pressure, Dr P Krishnaswamy, Professor of Therapeutics in this college, kindly undertook to make the observations recorded in Table I on the patients in his wards at the Madras General Hospital.

GRAPH 1

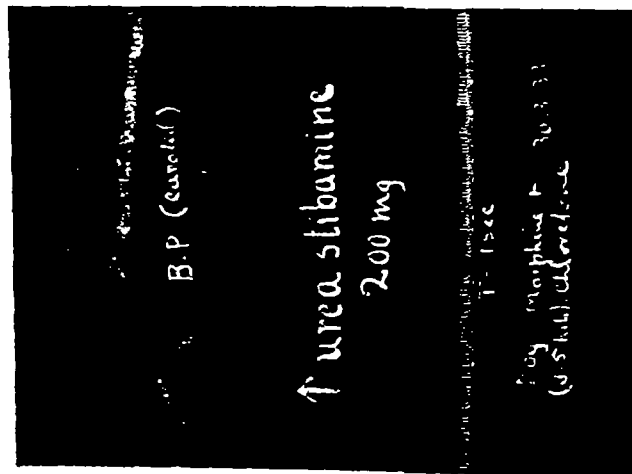


Fig a

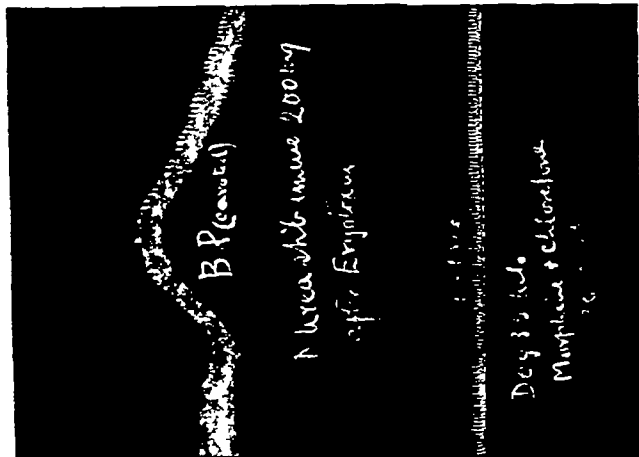


Fig b

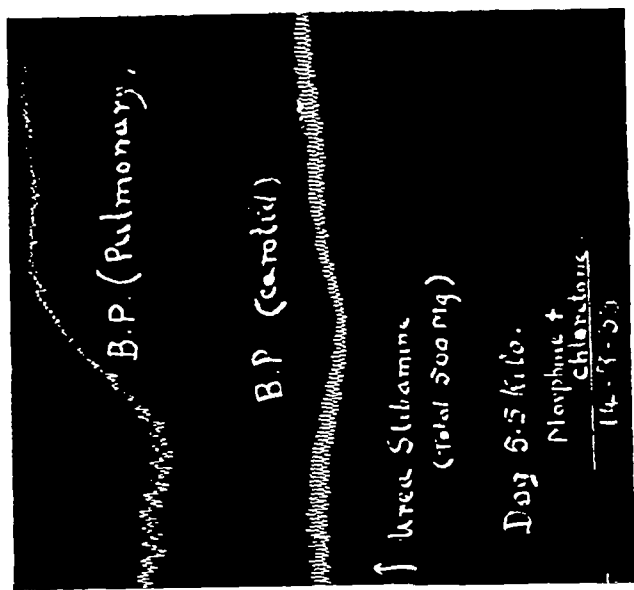


Fig c

GRAPH 2

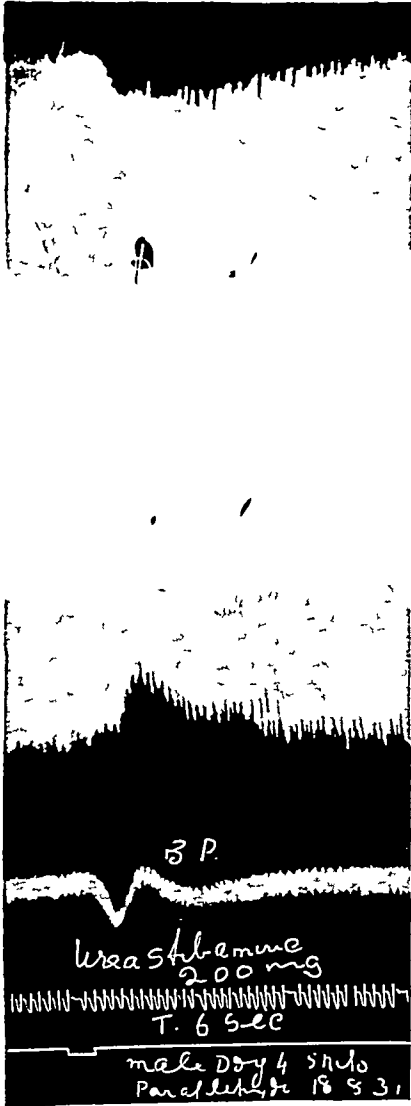


Fig. a

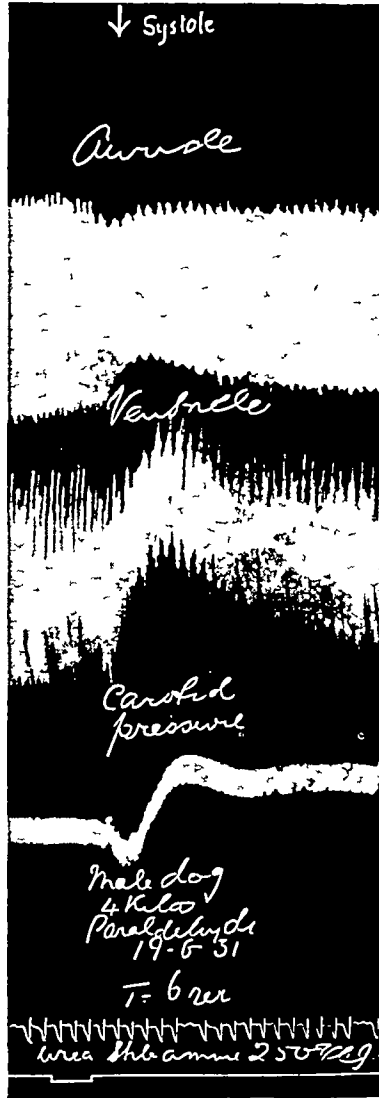


Fig b

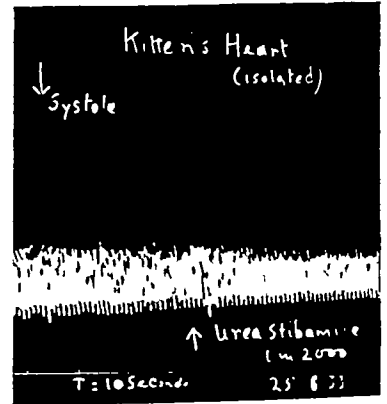


Fig c

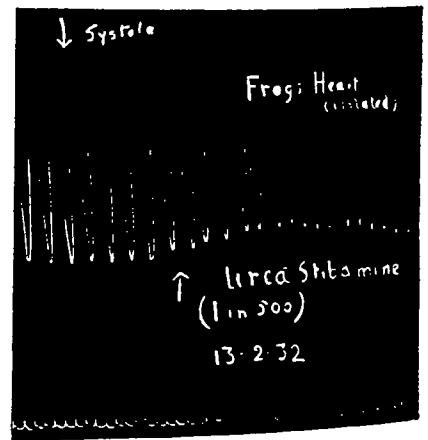


Fig d

GRAPH 3

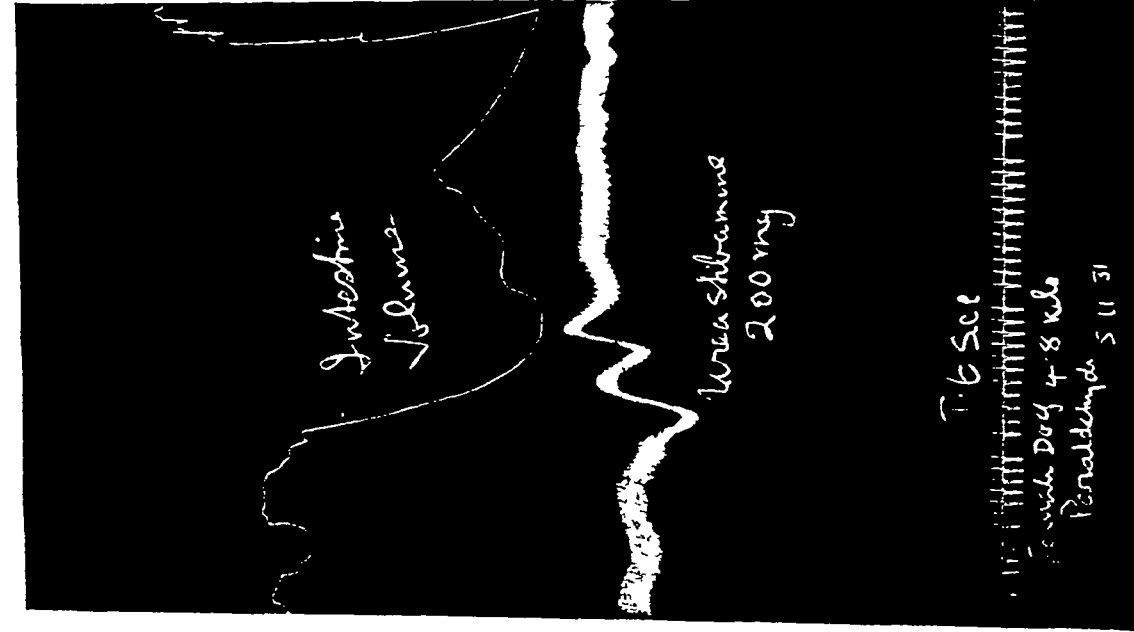


Fig a



Fig b

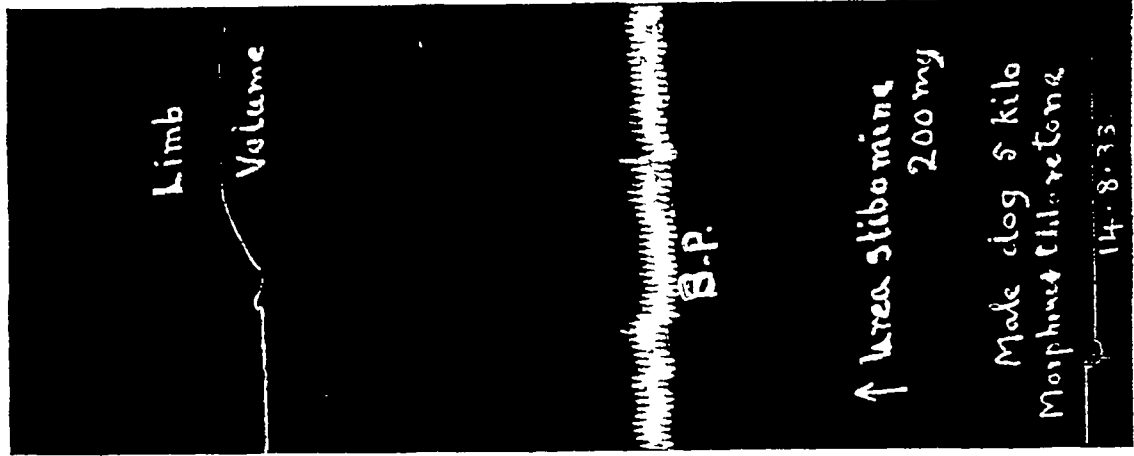


Fig c

TABLE I

Blood-pressure before and after urea-stibamine in man

Name	Quantity given in mg	BLOOD PRESSURE BEFORE INJECTION		BLOOD PRESSURE AFTER INJECTION	
		Systolic, mm	Diastolic, mm	Systolic, mm	Diastolic, mm
Kasi	50	104	60	96	52
Said Ali	100	100	70	105	75
Venugopal	100	115	85	105	70
Ethirajulu	100	114	68	110	58
Arogyasamy	200	116	68	112	66
Venugopal	200	110	68	110	68
Palayam	200	105	60	105	65
Apthajothi	200	115	60	110	70
Ramasami	200	130	65	130	50
Arulsami	200	115	60	110	70

The results show that the effects, if any, on both systolic and diastolic blood-pressure are inconspicuous. There was an average fall of 6 mm of systolic blood-pressure in 6 of the ten cases, no change was noticed in 3 and the blood-pressure rose by 5 mm in one instance.

Oncometric records were taken of the volumes of the spleen, intestines, kidney and the hind limb. The limb volume showed an appreciable rise (Graph 3, fig c) but the volume of the spleen and the intestine (Graph 3, figs a and b) invariably showed a very well-marked fall. The kidney volume also was reduced.

We perfused these organs removed from the body, using the perfusion apparatus of Dixon at body temperature. Our method was to anaesthetize the animal with ether or chloroform and then bleed it through the carotid artery, at the same time injecting saline through the femoral vein. When the fluid coming out of the carotid cannula became pale, the animal was opened up and the organ removed. This served to wash out the blood from the organs and prevented blood clotting inside. A cannula was placed in the main artery, any collateral branch was ligatured and the organ placed over a Buchner's funnel inside the Dixon's apparatus. The perfusion was then started with oxygenated Locke's fluid containing defibrinated blood from the same animal. The stroke of the pump was adjusted to a convenient height so as to deliver on an average about 0.5 c.c. per stroke. The perfusing fluid flowed out of the organ through the vein and was caught in the graduated vessel provided for the purpose. The time taken for the outflow of 50 c.c. was recorded by means of a stop-watch and when several readings were more or less constant, a dose of urea-stibamine was injected into the rubber-tubing just above the arterial cannula. The time taken for the outflow of 50 c.c. was again recorded, several readings being taken in order to obtain the maximum effect. Experiments were done in this manner with the following isolated organs: liver, spleen, kidney,

the hind limb and the lungs. In the case of the latter, both lungs with the trachea and the heart were removed. The arterial cannula was introduced into the opening of the pulmonary artery in the right ventricle, the outflow being from the left auricle, the rest of the heart was snipped off. The lungs were moderately inflated and deflated by means of Schuster's artificial respiration pump. In perfusing the limb, cannulae were placed in the femoral artery and the saphenous vein and the limb disarticulated. Ligatures were placed around any bleeding point.

Table II gives the results of our experiments —

TABLE II

Organ perfused	Dose of urea-stibamine in milligrams	Average time for outflow of 50 c c before injection	Maximum retardation after injection for 50 c c
		mins secs	mins secs
Liver	10	9—42	5—26
	10	12—59	7—0
	10	5—26	3—19
	25	6—16	5—49
	30	3—53	3—0
	48	3—20	3—25
Spleen	10	5—26	4—17
	20	6—2	10—6
	25	6—50	3—10
	25	11—45	2—19
Kidney	20	7—13	7—49
	25	6—20	4—30
	25	13—37	19—0
Lungs	25	2—7	3—32
	25	2—54	1—6
	48	7—20	2—35
	50	2—33	0—48
Limb	25	4—25	1—49
	50	2—35	2—35
	50	7—50	13—0
	50	4—30	1—30
	50	3—33	1—2

The outflow from the liver was markedly delayed with as small a dose as 10 mg, the maximum delay noticed being 7 minutes. It would seem that as much retardation has been obtained with 10 mg as after as high a dose as 48 mg and in two of the recorded cases the delay was even greater with the smaller dose. This retardation of outflow was a constant feature. 0.01 mg of adrenaline slowed the outflow to a very considerable extent, a delay of as much as 24 minutes having been noticed in one case. It is interesting to note the effect of injecting histamine. Three mg

to five mg caused a transient quickening followed by retardation of outflow in two experiments. But larger doses of 5 mg to 10 mg constantly slowed the rate of outflow. In one case where the liver was continuously perfused with Locke's fluid containing 0.1 per cent of histamine a retardation was the only result observed.

Under identical conditions neostibosan and tartar emetic both caused restriction of outflow from the liver. In the case of the former, in two experiments the maximum retardation was 48 seconds with 50 mg and with the latter 1 minute and 15 seconds with 35 mg. Neostibosan has but little effect on the blood-pressure, there may be a slight fall sometimes. Tartar emetic always causes a fall of blood-pressure due to a more marked action on the heart. No secondary rise of blood-pressure ever occurs with either.

Perfusion of the isolated spleen with various strengths of urea-stibamine showed retardation of outflow. Small doses produced just a transient delay in the outflow. In one instance, 10 mg first slowed the rate of outflow by 31 seconds and the outflow almost immediately returned to normal. Twenty mg or more caused a constant retardation of outflow.

Identical results were obtained with the kidneys, 20 mg or more producing a constant restriction of outflow from the renal vein. In one case, 12½ mg produced no effect while a further injection of 12½ mg slowed the rate of outflow by as much as 19 minutes.

The lungs also showed a retardation of outflow but the effects were certainly weaker. The maximum retardation of outflow noticed was only 3 minutes 32 seconds and even with big doses of about 50 mg, marked slowing was never obtained. Addition of about 0.01 mg of adrenaline to the perfusing fluid first caused an acceleration of the outflow and this was followed by constriction.

The effect of perfusing the isolated hind limb of the dog was not as constant. Large doses were needed to produce an appreciable effect. In only one case was the retardation of the outflow from the vein marked, it was 13 minutes, with 50 mg. In the other experiments, the maximum retardation noticed was not more than 2½ minutes. In two cases there was an initial slowing followed by a quickening of the outflow (see Table III).

TABLE III

Organ perfused Hind limb

Drug used	Dose mg	Average time for outflow for 50 c.c before injection	Maximum retardation of outflow	Maximum subsequent acceleration
		mins secs	mins secs	mins secs
1 Urea stibamine	50	9—36	4—19	4—17
Adrenaline	0.02	1—30	1—30	1—35
2 Urea stibamine	20	4—25	0—49	0—43

Adrenaline too produced an initial retardation of outflow followed by acceleration. In a single experiment, the limb *in situ*, i.e., with the nervous connections intact, was perfused with histamine. One mg caused a quickening of the outflow by 1 minute 49 seconds. There was no preliminary slowing.

DISCUSSION

Urea-stibamine given intravenously to anæsthetized dogs more often produces a preliminary fall of blood-pressure followed by a rise above normal. This subsequent rise is not abolished by ergotoxin. From the experimental data given above, we are led to believe that the primary fall of blood-pressure is due to a temporary depression of the heart muscle, causing a transient dilatation of the chambers and a diminished cardiac output. The cardiac effect is but fleeting and so the blood-pressure quickly returns to normal. The subsequent rise of blood-pressure we ascribe to a constriction of the blood vessels of the splanchnic area as well as those of the kidneys. This is to a certain extent compensated for by a moderate dilatation of the surface blood vessels. The volumes of the spleen, intestines and kidneys show a marked decrease. Perfusion of the isolated organs, viz., the liver, the spleen, the kidneys and the lungs, all indicate a constriction of the blood vessels in these organs leading to a diminished outflow of the perfusion fluid from the veins. The liver and the hind limb were perfused with histamine which is known to be a capillary paralyzant. In the case of the liver the outflow was diminished but in the case of the hind limb acceleration of the outflow was observed. According to Samson Wright histamine causes dilatation of both arterioles and capillaries in the dog and thus explains the vasodilatation of the surface vessels and the consequent quickening of the outflow after histamine. The apparent similarity of action of both urea-stibamine and histamine when perfused through the isolated liver needs some explanation. Bauer, Poulsson, Dale and Richards (1932) perfused the dog's liver with histamine and adrenaline. They found varying effects with different doses of adrenaline and observed that a relatively large dose of adrenaline, causing a pronounced constriction of both arterial and portal branches, would be accompanied by a diminution of outflow, while a subsequent smaller dose, producing a small rise of arterial pressure, would cause a definite increase of venous outflow. The liver volume showed a constant decrease after adrenaline. Histamine also produced a reduction of outflow, but it caused a swelling of the liver. According to these authors, in addition to the influence of the vasomotor mechanism, the outflow from the dog's liver is controlled by a sluice mechanism, situated near the caval orifices of the main hepatic veins, which is closed by histamine and opened by adrenaline. Hence the decreased output after histamine while a succeeding small dose of adrenaline opens the sluice and lets the accumulated blood to escape. We were unable to obtain a satisfactory record of liver volume, but since the volume of the spleen and the intestines decrease there is no reason to believe that the liver volume would behave in a different way. On the other hand it has been shown to be increased after histamine. Therefore, the restriction of outflow after urea-stibamine must be put down to a constriction of the hepatic vessels. From the data given in Table II it will be seen that the liver vessels are most sensitive to the action of urea-stibamine, small doses readily causing a constriction, while the lungs show a minimal effect even with large doses.

Chopra and Das Gupta (1928) found that provocative injections of urea-stibamine and other antimonials liberate the Leishman-Donovan bodies into the peripheral circulation. This they attributed to engorgement and increased rhythmic movement of the spleen and the liver producing a rupture of the endothelial cells so that the parasites appeared in the peripheral blood of these patients where they could not be detected before. Physicians have used injections of adrenaline for diagnostic purposes in both kala-azar and malaria in order to force the parasites into the peripheral circulation. This effect is to be attributed to adrenaline causing a marked constriction of both the liver and the spleen and thus squeezing out the parasites into the peripheral circulation. Urea-stibamine may conceivably act in a manner similar to adrenaline. The pulmonary pressure rises markedly after urea-stibamine (Graph 1, fig c). This would lead to a damming back of the blood in the right side of the heart and may partly be responsible for the temporary dilatation of the heart. The increased peripheral resistance may also under certain conditions contribute to a dilatation of the heart to start with. Such an effect has often been noticed when intravenous injection of as small a dose as 0.025 mg of adrenaline is given to anaesthetized dogs. It may be that the marked constriction of the splanchnic vessels noticed after urea-stibamine also may thus contribute to the temporary weakening and dilatation of the heart.

It has been observed that large doses, e.g., 50 mg, are required to produce an appreciable constriction of the blood vessels of the perfused isolated limb. In two of the experiments the initial effect was a vasoconstriction but this quickly gave place to a dilatation of the vessels resulting in acceleration of the outflow. Adrenaline also was found to produce identical effects. In the intact animal adrenaline constricts the splanchnic vessels and at the same time there is a compensatory dilatation of the peripheral and surface vessels. Similar results have been obtained after urea-stibamine. While adrenaline produces these effects by a sympathomimetic action, urea-stibamine perhaps acts more peripherally on the musculature as the pressor effect is not abolished by ergotoxin and as constriction of the pulmonary vessels is also noticed.

Antimony is usually classified under capillary poisons along with arsenic and histamine. The picture presented above does not seem to bear out this statement. The action of antimony on the circulation seems to be quite different from that of histamine. We gave intravenous injections of potassium antimony tartrate in 2 per cent solution to two dogs, starting with 1 c.c. Both died, one after 6 c.c., and the other after 18 c.c. They showed symptoms of weakness, apathy and had frequent motions prior to death. The motions were more of a dysenteric nature with slime and blood, but were not diarrhoeal. No typical rice-water stools were to be seen, as after arsenic. Post-mortem examination showed extensive necrosis of the liver and kidney with a certain amount of necrosis of the heart muscle. The stomach and intestines were not distended with any kind of fluid. Two rabbits were given urea-stibamine injections intravenously starting with 0.05 g. One died the day following the first injection and the other on the ninth day after 0.35 g were given. The animals did not show any gastro-intestinal symptoms. Only gradually increasing weakness was noticed. Post-mortem examination showed no indication of any gross internal lesion. Although we have not examined this point in detail we are forced to the conclusion that more

work will have to be done before antimony is established as a capillary poison, since antimony does not seem to behave like the capillary dilators, arsenic or histamine

SUMMARY AND CONCLUSIONS

- 1 The cardiovascular action of urea-stibamine on the dog has been investigated
- 2 Urea-stibamine produces marked constriction of the volumes of the spleen, liver, kidney and intestines. There is a compensatory increase of the limb volume. The vasoconstriction is most marked in the liver and least in the lungs
- 3 Two hundred mg to two hundred and fifty mg of urea-stibamine given intravenously to anæsthetized dogs leads to a transient depression of the heart and dilatation, as observed by the myocardiograph. This effect appears in about 40 seconds and disappears in $1\frac{1}{2}$ minutes
- 4 On the blood-pressure the more constant effect is a preliminary fall succeeded by a rise above normal. This rise is not abolished by ergotoxin and seems to be correlated with the marked constriction of the splanchnic blood vessels. The preliminary fall is perhaps due to the transient depression of the heart and diminished cardiac output. These results are obtained with doses that produce a concentration in the dog's blood of approximately 10 times that usually found in the human blood after therapeutic injections of about 200 mg of urea-stibamine
- 5 Electrocardiograms taken after intravenous injections of 200 mg of urea-stibamine to dogs and human beings show no abnormality
- 6 Therapeutic doses of about 200 mg given intravenously to human beings produce little effect on the blood-pressure
- 7 The classification of antimony among capillary poisons is questioned

Our acknowledgments are due to Dr P Krishnaswamy, Professor of Therapeutics in this college, and his assistants for the observations on the blood-pressure of patients under treatment for kala-azar, to Dr Totadri of the Government X-ray Institute for taking the electrocardiograms, to Dr A N Goyle, Professor of Pathology, and his staff for help in post-mortem examination of tissues and to Dr V Iswariah for help in the earlier stages of our experiments

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AN EPIZOOTIC AFFECTING LABORATORY-BRED GUINEA-PIGS

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THE outbreak was first noticed in a pen containing twenty-one young guinea-pigs from three weeks to three months old. Five of them were found to be affected by an acute type of conjunctivitis marked by severe swelling of the eyelids, apparently intense photophobia, and plenty of purulent exudate. All the five were at once isolated. Two days later three more from the same pen contracted the disease, and were isolated. A third batch of two more were attacked after another two days and this time the healthy ones were removed to another pen. Though there was one more attack in this new pen, the epidemic did not spread further for the time being.

An extreme quietness, staring coat especially marked about the head, and the hiding of their snouts under the belly of other animals were the obvious indications of the affection. On closer examination, the eyelids were found to be very much swollen giving the eye an appearance of standing out. The eyelashes had mostly fallen off and the skin around the eye was denuded of hair to a short distance. This portion of the skin was reddish white. Thick, copious, white discharge had collected at both the angles and formed a thin film over the eyeball. The conjunctiva was red and angry-looking and the cornea opaque. From the circumstances that the animal hid itself in corners or pushed its snout under the belly of other animals and from its trying to evade examination of the eye, there appeared to be extreme photophobia. Rhinitis was present in all the cases. The affection remained unilateral in many but in others it spread to the other eye. The affected animals were extremely ill and took their fodder very sparingly. Of the first batch of ten observed four died within the first five days of affection, presumably from a generalized infection and septicæmia. The temperature was not recorded, but the blood showed neutrophilia in two cases examined. The acute course of the disease lasted for five days. Four of the recovered animals were completely cured while the remaining two showed a residual chronic form of conjunctivitis.

An identical epidemic broke out two months later, nine young animals in a pen getting the affection. On both occasions the epidemic broke out during the rainy

season and only in those pens where the young guinea-pigs were housed, the grown up ones of the adjoining pens entirely escaping the infection

BACTERIOLOGY

The smears from the conjunctiva of these animals did not show anything elucidative. Material from the conjunctival sac was plated on nutrient agar and the organisms grown thereon were studied. Separate broth-cultures of four different types of colonies were made and a platinum loopful of each, after twenty-four hours' incubation, was instilled into the eye of different guinea-pigs with untreated broth in the other eye as control. It was soon found that one of the organisms isolated produced an identical type of acute conjunctivitis in the experimental animal in four to six days. Culture of the conjunctival material of this animal yielded the same organism. This was repeated several times successfully and every time a bacillus answering to the descriptions of the original one was isolated, thus satisfying Koch's postulates. Due to the resemblance this bacillus bore to the Gaertner's bacillus experimental inoculation with a stock culture of the latter also was done with negative results.

In the experimental animals the incubation period was four to six days decreasing by sub-passages to a minimum of four days, thus incidentally showing an exaltation of virulence by such passages. The experimentally produced disease in the adult guinea-pigs had a longer incubation period and was milder, often tending to chronicity. Attempts to infect rabbits were unsuccessful. An unsuccessful attempt was also made to produce an agglutinating serum.

The organism resembled the Gaertner's bacillus in many respects. It was motile and showed a tendency to pleomorphism. It was slightly shorter and thinner than the Gaertner's bacillus and was easily and uniformly stained by the ordinary aniline dyes. It was Gram-negative. No spore formation nor capsule formation was ever noticed. Broth cultures showed a uniform turbidity, the colonies on agar were also similar in appearance to the Gaertner colonies, but somewhat larger. It produced acid and gas in glucose, dulcitol, mannitol, maltose, galactose and sorbitol but not in lactose, salicin and adonitol. The litmus milk turned slightly acid in twenty-four hours but showed production of alkali on the succeeding two days. Indol formation was not a constant feature.

My grateful thanks are due to Dr C Ramamurti, B A, M B, B S, B S Sc, Professor of Bacteriology, Medical College, Vizagapatam, for permitting me to publish the above observation.

A STUDY ON THE PARASITES OF KALA-AZAR AND THEIR DISTRIBUTION IN THE BODY

BY

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INTRODUCTION

SINCE the discovery of the *Leishmania donovani*, very little scientific study had been made regarding the pathological changes and the distribution of the parasites in the various tissues of the infected patients. Christophers (1904) first took up this study in India and published his results in the 'Scientific Memoirs of the Government of India'. He definitely showed that the parasites of kala azar were found abundantly inside large swollen 'endothelial cells'. Later on, this subject was studied by Laveran (1909), Perry (1922), Meleney (1925), De (1927) and Cash and Hu (1927). A considerable advance was made by Meleney in his study on the experimentally produced disease in Chinese hamsters.

My observations are based on the study of necropsy cases obtained from the Medical College group of hospitals during the period of five years from 1927 to 1931. Only those cases in which the smears taken from the spleen after death showed *Leishmania donovani* are included in this series. The total number of cases studied was 26. In every case sections of the spleen, liver, bone-marrow, heart, kidney, intestines, supra-renal's, lungs and skin were made and examined.

The post-mortem examinations were made within the first six hours in 8 cases, between six and eight hours in 3 cases, between 12 and 24 hours in 9 cases and between 24 and 37½ hours in the remainder. In four cases decomposition had advanced too far to allow complete examination.

TECHNICAL METHODS

Tissues were fixed in Zenker's fluid, Helly's fluid, 10 per cent formol-saline, and methyl alcohol. One of the secrets of success in the successful demonstration of the parasites lies in freshness of the materials and perfect fixation. In my experience blocks of tissues should not be more than 2 mm in thickness before putting them into the fixing fluid.

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The most satisfactory demonstration of the parasites in tissues is obtained by a simple rapid method, described by the present writer (De, 1924a, 1924b). In this method, fixation is done by passing thin slices of tissues through several changes of methyl alcohol or, better still, acetone. Sections are cut after rapid paraffin embedding. These are stained either with diluted Giemsa's or Leishman's stain, like an ordinary blood film. Differentiation requires some experience, but if it is done carefully and quickly, under the low power of the microscope, with acetone, the parasites come out very sharply. Although the *Leishmania donovani* show up brightly and without much diminution in size, the general morphology of other cells and the histological structure of the tissues cannot be studied in detail owing to a large amount of shrinkage and consequent production of artifacts.

Another method that I have found very successful for this study is a modification of the method used by Wollbach (1922) in the study of Rickettsia bodies in Rocky Mountain spotted fever. The actual technique followed by me is as follows —

Fix thin slices in Zenker's fluid for 24 hours

Wash in running water for 24 hours

Pass through 70 per cent, 80 per cent and rectified spirit, absolute alcohol (two changes), and acetone. The whole process should not take more than 24 hours.

Clear in xylol or chloroform or, better still, in cedar-wood oil and block the tissues after rapid paraffin embedding.

The less the time required for the above, the brighter the picture.

Sections should not be more than 6 μ to 10 μ in thickness.

Remove paraffin as usual.

Remove corrosive sublimate by treating sections with Lugol's iodine, absolute alcohol, rectified spirit, 80 per cent alcohol, 70 per cent alcohol, and then put them in running water.

Remove any excess of iodine by 0.5 per cent hyposulphite solution for 5 minutes and then washing in running water for 10 to 15 minutes. They are finally washed in distilled water for 10 to 15 minutes after which they are ready for staining.

Stain in dilute Leishman's or Giemsa's stain, preferably overnight.

Formula for the Giemsa's solution

Stock Giemsa's solution	2.5 c c
Methyl alcohol	2.5 c c
Distilled water	100 c c
0.5 per cent sodium carbonate solution	5 drops

The stain should be prepared fresh every time. The sections should pass through three changes of the above stain, viz., half an hour in No. I, half an hour in No. II, and overnight in No. III.

Wash in distilled water.

Differentiate in 96 per cent alcohol with a little colophonium in it.

Rapidly wash in absolute alcohol.

Clear in xylol and mount in euparal.

It is better to differentiate under the microscope in successive stages with absolute alcohol or acetone, and wash in distilled water so that over-decolorization may be prevented.

In this way, the parasites as well as the tissues are beautifully stained and the morphological structure of the various cells can be studied with accuracy.

If one wants to ascertain their presence only, and the morphology of the parasites is not required in detail, the best method of staining is that by iron-haematoxylin and eosin after fixation by Zenker's or Helly's fluid. One great advantage in this method of staining is that the parasites may be detected quite a long time after death even when the tissues have undergone much post-mortem decomposition. In one of my cases, in which the post-mortem examination was done 37½ hours after death the parasites could be seen although very poorly.

Material fixed in 10 per cent formol-saline is not suitable for demonstration of parasites although the latter show as black specks when stained by iron-haematoxylin method.

DISTRIBUTION OF PARASITES

In 25 out of 26 of the cases investigated, parasites were found in the spleen, the liver, and the bone-marrow, in the 26th case decomposition had progressed too far for satisfactory examination and it was only possible to demonstrate them in the spleen.

In the *liver*, the parasites are located in the littoral cells of Kupffer, in surprisingly large numbers, causing an enormous increase in the size of the cells. The parenchyma cells of the liver are free from them. In cross sections some of the enlarged Kupffer's cells may resemble the hepatic cells very closely and may be easily mistaken for them. The number of cells containing parasites are small in the central zones where the capillaries are much dilated and both the Kupffer's and the hepatic cells are more or less atrophic, but towards the portal zone the parasites and their cell-hosts increase in number. In some cases, a few cells with parasites in them may be seen in the portal spaces amongst the collections of mononuclear cells which are found in the livers of patients who have suffered from dysentery and other intestinal diseases (Plate XXXI, fig. 1).

In the *spleen*, the parasites are found in large numbers inside large swollen mononuclear cells which are situated in the pulp tissue of the organ. There is a considerable increase in size and number of these reticulo-endothelial cells and, as a result, the limits of the Malpighian follicles are encroached upon by the pulp which in some cases extends from one arteriole to another, completely replacing the lymphocytic elements. In such cases, the substance of the organ consists of little else than blood sinuses and parasite-laden mononuclear cells and thus the characteristic splenic pattern is lost (Plate XXXI, fig. 2). The number of parasites in such a case may be so large that a section of a spleen resembles a film of a staphylococcus culture (Plate XXXII, fig. 3). In less severe cases, some portion of the lymphoid follicles remains but few parasites can be seen there although the pulp tissue is full of them (Plate XXXII, fig. 4). I have not been able to find any parasite inside the true endothelial cells of the capillaries and blood sinuses, but many giant mononuclear cells heavily laden with parasites may be seen in the lumen of the

blood spaces as free elements (Plate XXXIII, fig 5) Besides this, many such parasite-laden cells are to be found as free elements in the lumen of the arterioles and veins which are cut longitudinally, in association with the other elements of blood clot (Plate XXXIII, fig 6)

In the *bone-marrow*, an almost similar picture is found Many parasites, all intra-cellular, are seen inside similar kinds of cells which vary in number according to the intensity of the infection (Plate XXXIV, fig 7) The yellow marrow may be replaced, in varying degree, by red marrow where similar cells and parasites abound

The distribution of the parasites in other parts of the body is very inconstant My observations are as follows —

Lymphatic glands —Although the cells of the reticulo-endothelial system are known to be fairly abundant in and about the sinuses, the parasites are, as a rule, scanty or absent Occasionally, a few large parasitized cells may be seen in the big lymphatic spaces after a very prolonged search of several sections These are the usual blood histiocytes which have phagocytosed the parasites In only one case were parasites found—they were in small numbers and lying intra-cellularly in the glands which had been draining an inflamed area, viz, the gums and mouth

Gastro-intestinal tract —I have searched various parts of the tract for parasites very thoroughly in order to corroborate the observations of Christophers, Perry, Banerji (1923) and Meleney The entire gastro-intestinal tract was searched for granulomatous areas as suggested by Christophers or for the characteristic hypertrophic villous prominences, mentioned by Perry, but I could not find any Pieces from the stomach, duodenum and every foot of the small and large intestines were taken for histological study Careful search failed to reveal any parasites in these tissues One disadvantage, however, in this study has been the post-mortem decomposition which had set in in many of these organs owing to delay in holding the necropsy examination

Supra-renal gland —Parasites were occasionally found in the cortex of the supra-renal gland In this organ, the leishmania were seen in the mononuclear cells lying between the cells of the zona fasciculata and the zona glomerulosa, but none in the zona reticularis (De, 1924a, 1924b) In some cases, a focus of round-celled infiltration was found in the cortical portion, and in the inflammatory cell mass a few mononuclear cells containing parasites could be seen Out of 26 cases studied, I found two such instances where parasites were detected, but only in small numbers, (Plate XXXIV, fig 8)

Heart —In only one case were parasites found in the substance of the heart This was in a patient who had died of kala-azar with secondary infection by *Bacillus tuberculosis* An area of inflammatory reaction was present in the substance of the heart and a few parasites were found inside some of the mononuclear cells which accumulated in that area Otherwise, no abnormality could be detected and no parasites could be seen in any other part of the organ The parasite-laden mononuclear cells may be seen in blood clots inside the heart, in the veins and in dilated blood spaces practically everywhere in the body This is of course to be expected in a malady wherein *Leishmania donovani* can be demonstrated in peripheral blood

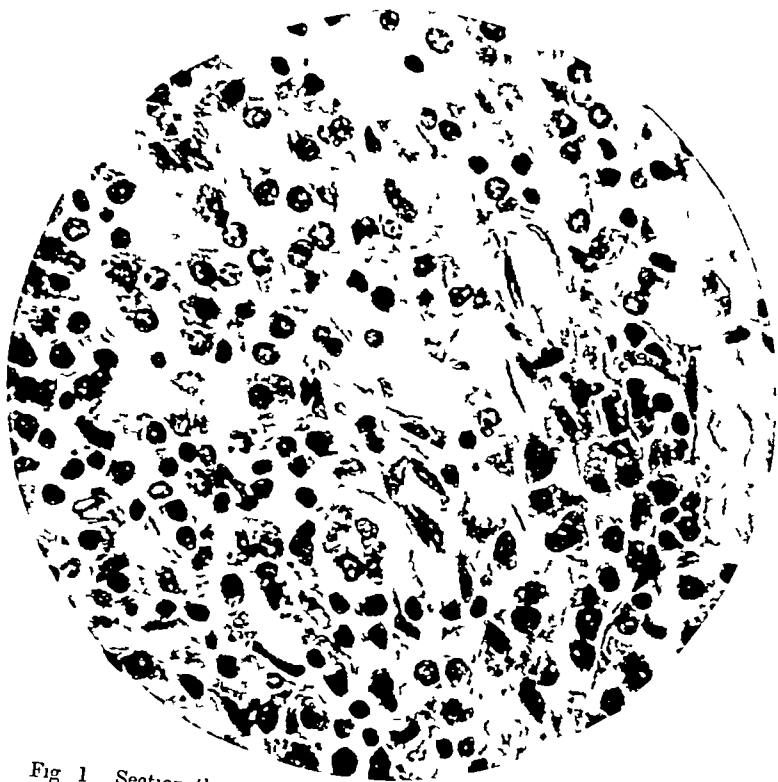
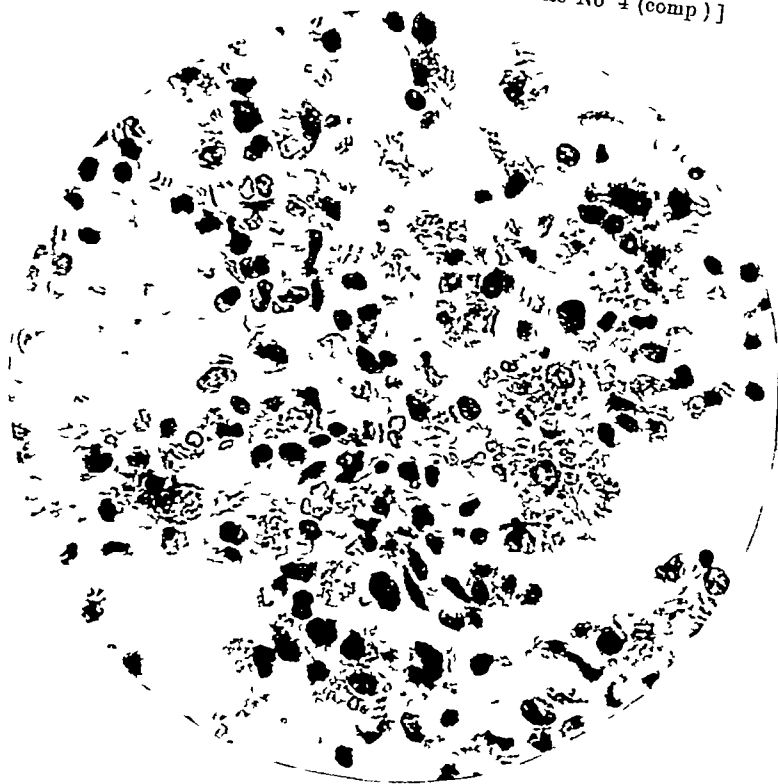


Fig 1 Section through the portal space of the liver showing a large accumulation of round cells and only small number of parasites
[Objective—oil immersion 1/12, Ocular—Zeiss No 4 (comp)]



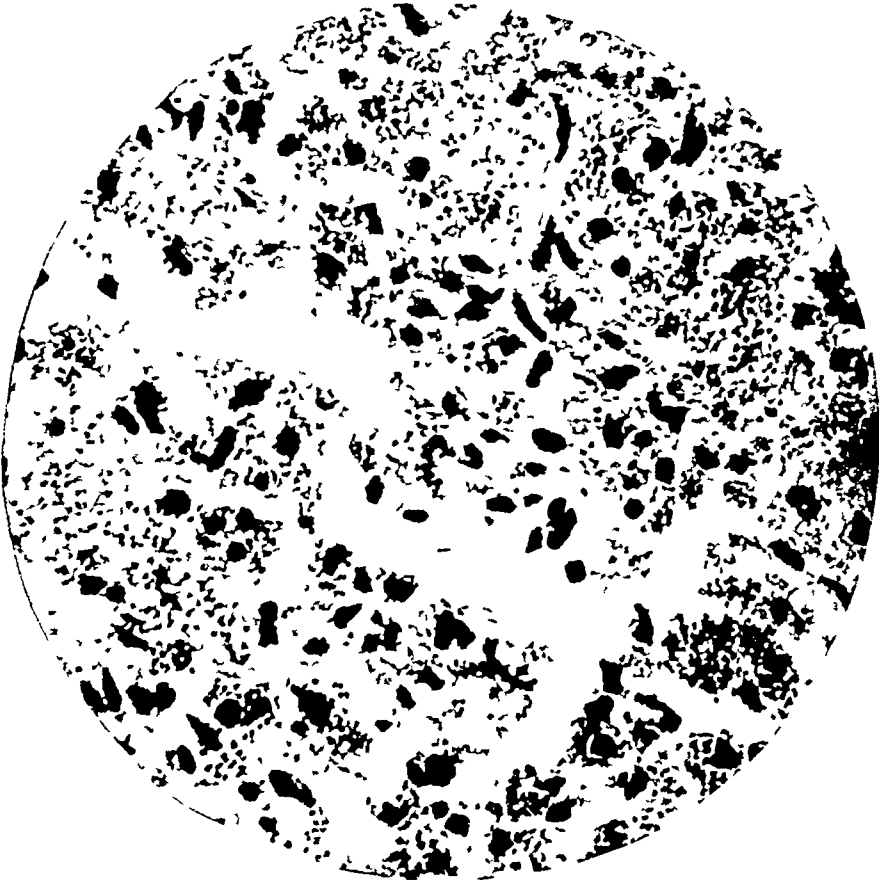


Fig 3 Section of a heavily infected spleen showing enormous number of parasites Their actual number is larger than what is shown in the figure, as all of them could not be brought under the same focus (Objective—oil immersion 1/12, Ocular—Leitz No 8 periplanatic)

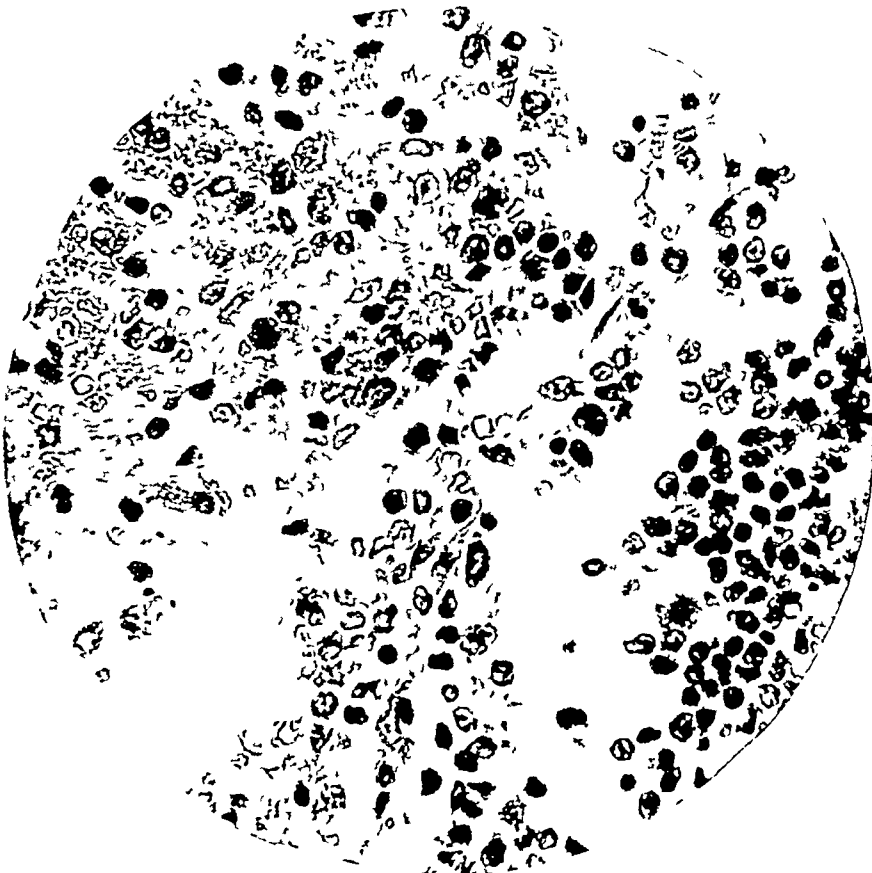


Fig 4 Section of spleen showing no parasites in the Malpighian follicle though the adjacent pulp is full of heavily infected cells (Objective—oil immersion 1/12, Ocular—Leitz No 8 periplanatic)

PLATE XXXIII

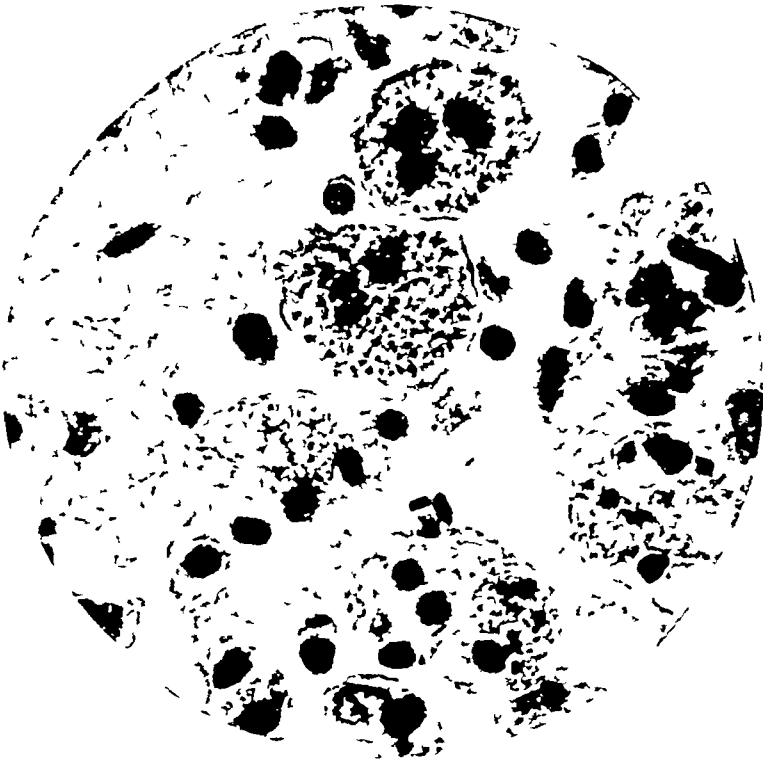


Fig 5 Section of spleen showing heavily parasitized giant mononuclear cells in the blood spaces
(Objective—oil immersion 1/12, Ocular—Leitz No 12 periplanatic)

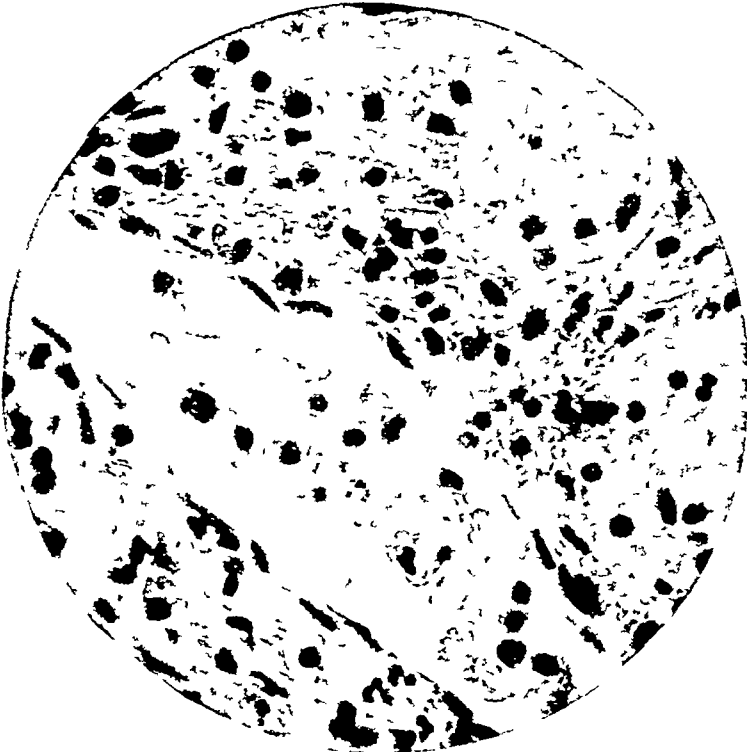


Fig 6 Section of spleen showing longitudinally cut venule containing many parasitized mononuclear cells. The endothelial lining does not show any change nor do they contain any parasites
[Objective—oil immersion 1/12, Ocular—Zeiss No 4 (comp)]

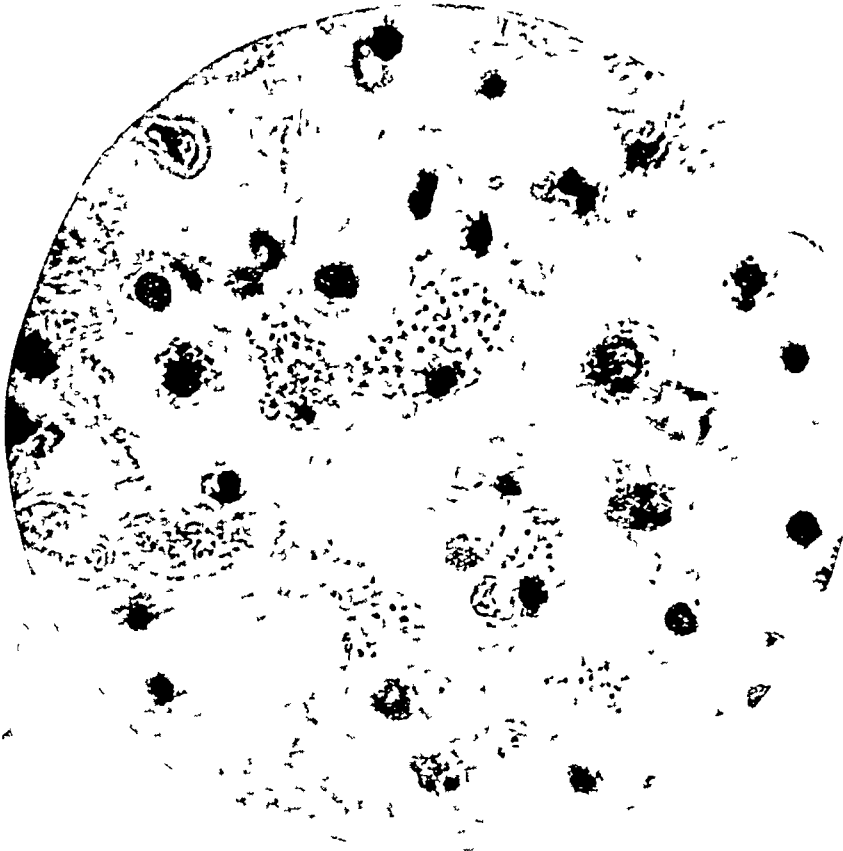


Fig 7 Section of bone-marrow showing parasitized cells and cavity formative tissue
(Objective—oil immersion 1/12, Ocular—Leitz No 12 periplanatic)

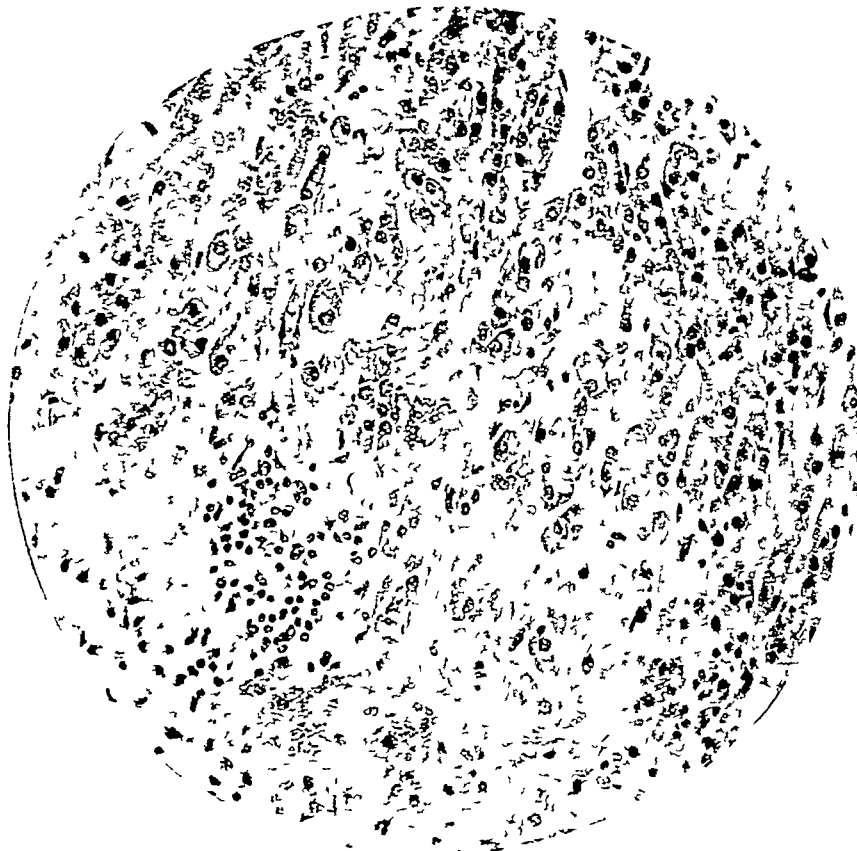


Fig 8 Section of supra renal gland showing a focus of round-celled infiltration in the zona fasciculata where a few parasitized cells were detected
(Objective—high power apochromatic 3 mm, Ocular—Zeiss No 4 periplanatic)



Fig 9 Section of skin from a case of dermal leishmaniasis showing many parasites and a heavily parasitized multinucleated giant cell
(Objective—oil immersion 1/12, Ocular—Leitz No 12 periplanatic)

Lungs—In the lungs, the parasites are as a rule very scarce or absent. Occasionally, in a very heavily infected case, one can see a few reticulo-endothelial cells containing them, situated around the bronchioles and intimately associated with the carbonaceous pigments. Unless care is taken, the carbon pigment may entirely overshadow the parasites which will thus escape detection. In cases of pneumonia, pulmonary tuberculosis or other lung diseases, it is impossible to locate the parasite-infected cells owing to preponderance of inflammatory exudate and fibrosis. I found only one case in which leishmania were present in such a position.

Skin—The presence of a large number of leishmania in the skin, in human cases of kala-azar, is described by workers in China, Cash and Hu (1927), and in experimentally inoculated hamsters as described by Meleney (1925), could not be corroborated by me in normal cases of kala-azar as found in Bengal, although some of the cases were heavily infected. I did not see any nodular cutaneous eruption as described by Christophers in two out of his seven cases. While this is true of uncomplicated cases of kala-azar, the picture is very different in dermal leishmaniasis, in these cases, the nodular eruptions show large numbers of mononuclear cells packed with the parasites filling up the inter-papillary areas in the corium (Plate XXXIV, fig 9).

COMMENTS

It will be seen from the above descriptions, that the parasites of kala-azar appear to have a special affinity for a special group of cells which abound in the liver, spleen and bone-marrow and that they are present in small numbers in other parts of the body. These cells have a common morphology and are included in the group of cells classified by Aschoff (1924) and Kiyono (1914) as the reticulo-endothelial cells, and by Ranvier as clasmatocytes. One of the many functions of these cells is the storing of pigments or of any foreign particulate matter introduced into the system either artificially, as by vital staining, or naturally in the process of infection. These parasites cannot remain in the blood as freely circulating bodies for any length of time as they are either destroyed by the leucocytes of the body or else phagocytosed and taken up by the reticulo-endothelial cells. Another observation confirmed by the above study is that, although the leishmania are so readily ingested by the phagocytic cells, they do not appear to be killed or digested readily, as may be judged from the intact morphology and the perfect staining reactions retained by the parasites, at all stages of the infection. Not only are these bodies uninjured, but they also appear to undergo multiplication while inside the protoplasm of the reticulo-endothelial cells.

It is difficult to explain the paucity in the number of the parasites in other parts of the body apart from the liver, spleen and bone-marrow. One of the reasons why they are so scanty or absent in these areas may be the enormously increased size of the host cells which are too big to pass through the narrow capillaries. Besides, when parasites escape, following the disintegration of some of these cells, they are immediately taken up by the reticulo-endothelial cells and also by the leucocytes of the circulating blood. Although the leucocytes, including the large monocytes which belong to the reticulo-endothelial system, circulate through every part of body, they do not under ordinary conditions come out of the circulation to discharge the parasites inside the tissues where the reticulo-endothelial cells are

present. The parasites either die out or these leucocytes manage to kill them and dispose of their loads by enzymatic action. Thus, the clasmatoocytes of the tissues do not get an opportunity, in the normal course of events, to come in contact with the parasites and to ingest them. Hence the absence or scarcity of leishmania in other parts of the body.

Christophers (1904), Cash and Hu (1927), Perry (1922) and others have, however, definitely found parasites in the skin, intestine and other areas where I failed to find them, but they did not find them constantly in every case. By studying very minutely the descriptions given by these workers and comparing them with my own observations, I find one important factor present in their cases, viz., the presence of some inflammatory reaction in the areas concerned. By producing an area of inflammation by artificial means and, at the same time, using vital staining Macklin and Macklin (1920), Russel (1929), and Carmichael (1929) have obtained the characteristic staining even in places where they could not succeed by employing the usual method. The stimulus of an inflammatory reaction helps to bring into intimate contact the vital stains and the stimulated reticulo-endothelial cells, which had been lying there in a dormant state, the pigment being derived partly from the effused blood and partly from the out-wandering blood phagocytes which have ingested it.

It is reasonable to suppose that a similar phenomenon takes place in leishmania infection and the occasional presence of the parasites in the various abnormal situations, as noted above, can be readily explained on the results of these experimental studies. It is possible that the inflammatory reaction, set up by some irritant in these tissues, determines the presence of the parasites in that area. Whenever there is an inflammatory reaction in any tissue, the vessels dilate and the wandering cells (which belong to the group of reticulo-endothelial cells) aggregate in large number and many leucocytes, including polymorphonuclear and large mononuclear cells, emigrate from inside the blood vessels into the site of irritation. If such a reaction takes place in the tissues of a man who is already infected with *Leishmania donovani*, some of the leucocytes which might have already ingested the parasites will migrate to the place of irritation, carrying the latter with them inside their cytoplasm. The dilated blood vessels may also allow some of the swollen parasitized cells to pass through them and reach the area. Subsequently when some of these cells die and disintegrate, they discharge the intra-cellular parasites at the site where the phagocytic mononuclear cells, the clasmatoocytes, have already gathered and which at once phagocytose them. Thus, when once the infection of this group of cells takes place, it is continued by rapid multiplication of parasites and proliferation and enlargement of the infected cells. It may be that the mild inflammatory reaction, which primarily started the process, completely disappears, by a process of resolution, leaving behind no traces. In that case, the subsequent picture will show nothing but an area containing many mononuclear cells with intra-cellular parasites just as one finds them in the liver or spleen. In time, the area of tissue in question will show nothing but a mass of the large parasitized cells—a sort of new formation consisting of these clasmatoocytes. These will readily appear to the naked eye as papillomatous or granulomatous nodules described by some of the pioneer workers. Such tumefactions may therefore be seen in the skin surface or in the intestinal mucous membrane, the commonest epithelial surfaces which are subject to irritation and inflammation due to traumatism or parasitic infection.

The regional lymphatic nodes, in which we cannot normally demonstrate the parasites, even in a very heavily infected case, will show them, as they drain the infected area from which parasites and the parasite-laden cells may reach them, via the lymphatic stream

In two of my cases, I found parasites in the supra-renal cortex in small foci of round-celled infiltration inside which the *Leishmania donovani* were present. In one case, the parasites were detected in a similar collection of cells in the substance of the heart muscle, and in another instance, in a case of broncho-pneumonia with stomatitis they were present in the sinuses of the enlarged cervical lymphatic glands. Christophers found them in sub-arachnoid and cerebral hæmorrhages and also in the edges of an ulcer in the intestines. He also found parasites in the skin ulcerations in the lower extremity and in the regional lymph glands of a patient dead of leishmaniasis infection. Banerji (1923) found them in the scrapings of ulcer in the stomach of a kala-azar case.

It is frequently stated that the parasites are found in the 'endothelial cells' lining the vascular channels. This I have entirely failed to corroborate. There are, in the literature, references to the phagocytic activity of the vascular endothelium; McJunkin (1928) succeeded in demonstrating pigment particles which were ingested by the vascular endothelium, particularly that of the newly formed capillaries of granulation tissue. Sabin, Doan and Cunningham (1925) are also of opinion that vascular endothelium is phagocytic, but Gardner and Smith (1927), Stilwell (1926) and Maximow (1927) all agree that vascular endothelium has no power of phagocytosis. It would appear that phagocytic activity of vascular endothelium has been demonstrated only under abnormal circumstances, as for instance, in newly forming capillary endothelium of granulation tissue, where the cells are not yet sufficiently differentiated. In view of the fact that vascular endothelium is considered to be a highly specialized tissue, very well differentiated functionally, I do not see why it should show power of phagocytosis. It is true that, embryologically, they are derived from the same stock from which the reticulo-endothelial cells originate and therefore should show power of phagocytosis, but in the process of development they become entirely differentiated and lose their original qualities except in the case of the liver capillaries where, in the littoral cells of Kupffer, this primitive power is retained to its fullest extent. My failure to find any parasites in the living cells of the vascular channels, except in the liver, tends to support this view.

SUMMARY

1 The different technical methods for the demonstration of the parasites in kala-azar and other leishmania infections have been described and their relative values discussed.

2 The parasites are always found in large number in the liver, spleen and bone-marrow, and only occasionally in the other parts of the body.

3 The parasites do not remain as freely circulating bodies, but are always inside large swollen phagocytic mononuclear cells—the reticulo-endothelial cells of Aschoff or the plasmatocytes of Ranvier.

4 Presence of parasites inside vascular endothelium could not be verified and an explanation has been given.

5 When parasites are present in areas other than the liver, spleen and bone marrow there is usually an inflammatory reaction excited by some cause. The relationship of inflammatory reaction to the infection of the reticulo-endothelial cells by leishmania has been discussed.

I offer my sincere gratitude to the Indian Research Fund Association for bearing the cost of this investigation. I am also very grateful to Colonel Sir Robert McCarrison, I M S, for his encouragement and valuable suggestions during my work. Lastly, I take this opportunity to express my great indebtedness to Dr L. E. Napier, in charge of the Kala-azar Department of the Calcutta School of Tropical Medicine and Hygiene, who has very kindly gone through my paper and given me much help and encouragement.

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STUDIES ON THE ANTIGENIC STRUCTURE OF *VIBRIO CHOLERÆ*

Part V.

ANALYSES OF *VIBRIO* PROTEINS NITROGEN DISTRIBUTION

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WE are analysing the protein fractions of the cholera and the cholera-like vibrios by two methods the nitrogen distribution method of van Slyke, and the racemization method The results obtained from the use of the first method on eight vibrio proteins are presented in this paper

From a consideration of the results of other workers, we believe that the comparative study of the vibrio proteins by these two methods will allow us to determine their identity or non-identity, both in composition and structure

METHOD

The organisms were grown on agar for 48 hours, washed off in distilled water, and killed by the addition of 0.5 per cent phenol The bacterial mass was centrifuged, and washed in distilled water Alternate washings and centrifugings were continued until a hydrolysed sample of the wash-water no longer reduced Benedict's solution Three or four washings were usually necessary before accomplishing the removal of the carbohydrate, which probably dissolves out of the agar during the period of incubation Failure to remove all the carbohydrate is followed by irregular results in the protein analysis (see the analysis of vibrio No 1716 in the Table) The washed protein was dried in an air oven at 50°C

TABLE
Nitrogen distribution in the protein fractions of cholera and cholera-like vibrios

Number — Agglutination reaction —	1612	1612 A	486	7812	1676	E	W 3075	W 880	1716*
	Agglutin- able	Agglutin- able	Agglutin- able	Agglutin- able	Agglutin- able	Agglutin- able	Non agglu- tinable	Non agglu- tinable	Agglutin- able
Characteristic sugar of the carbohydrate frac- tion —	Galactose	Galactose	Galactose	Galactose	Galactose	Arabinose	Arabinose	Arabinose	Galactose
Percentage of nitrogen									
Amide N	15.49	15.57	15.28	14.07	11.46	14.35	11.64	13.75	12.17
Humic N	6.30	6.09	7.00	6.88	6.86	7.16	7.22	6.69	7.21
Total bases	4.75	4.06	4.73	4.23	4.96	4.63	4.16	4.40	7.50
Arginine	24.20	26.03	25.70	24.30	21.84	21.20	24.80	21.08	21.71
Histidine	11.15	11.22	11.83	10.91	11.65	10.84	12.03	11.35	11.46
Lysine	5.00	5.72	5.26	4.27	5.28	5.33	3.11	3.27	7.23
Cysteine	8.05	9.09	8.61	9.13	7.91	8.04	9.66	9.46	3.03
Total mono amino acids	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mono amino N	63.19	61.47	63.45	61.54	64.41	63.30	63.49	64.89	65.63
Non amino N	55.09	56.83	55.94	55.56	55.64	55.58	54.84	56.64	57.11
	8.10	7.64	7.52	8.98	8.78	7.72	8.66	8.26	8.53
Total nitrogen	98.41	100.65	100.86	99.94	101.07	99.29	99.66	100.06	102.06

* See Text

Care was taken that the proteins should not be subjected to strong acid solutions or to high temperatures during their preparation. In each case (except that of vibrio No 1612-A where a single determination was made) the analysis was done in duplicate six grammes of protein being used. The figures given in the Table represent the average of the two determinations, which in every case agreed very closely.

Strain No 1612-A is the same as strain No 1612, save that it had undergone a further purification, as follows. A weighed quantity of protein of vibrio No 1612 was treated with ether at room temperature, allowed to stand for two days, and the ethereal extract removed. This process was repeated three times. The ethereal extracts were added together, and the ether removed by evaporation. The remaining material represented a fat content of the original of 2.05 per cent.

The defatted material was then dissolved in the least quantity of 1 per cent caustic soda, centrifuged to get rid of any insoluble impurities, and the protein precipitated with 2 per cent acetic acid. This process was repeated twice more. After two further washings with 2 per cent acetic acid, the material was washed twice with 95 per cent alcohol, and then twice with anhydrous ether, and finally dried *in vacuo* over sulphuric acid.

As a reference to the Table will show that this procedure did not cause any outstanding difference in the figures of the analysis. This further purification was therefore not continued.

RESULTS

In addition to the strains upon the carbohydrate content of which we have already reported (Linton and Shrivastava, 1933), four new strains are included in this analysis. Of these Nos 7812, 486 and 1676 are typical agglutinable vibrios derived from cholera. Their characteristic sugar is galactose. Water-vibrio No 880, derived from water in Calcutta, is non-agglutinable, and contains arabinose. Of the organisms used, five are galactose-containing and three arabinose-containing, of which one (E) is agglutinable, and derived from a case of cholera, and the other two are non-agglutinable water-vibrios.

The analysis of strain No 1716, an agglutinable, galactose-containing vibrio from a cholera case, is included in the Table, since it shows what irregular and contradictory results will be obtained in the van Slyke analyses unless the protein is carbohydrate-free. This effect, which is most marked on the humin nitrogen, and on the bases, has frequently been noted by previous workers. The close similarity in the figures of the other proteins shows, on the other hand, that the carbohydrate had been separated from them, at least to the same degree, in each case.

Our figures do not give any indication that any marked differences in nitrogen distribution exist among these vibrios. The only apparent dissimilarity lies in the somewhat lower histidine figures for the cholera-like vibrios, the other basic amino-acids, however, are similar throughout the entire group.

Figures obtained by the van Slyke analyses of other bacteria have been collected by Hirsch (1931) from various papers, and published in connection with his own analyses of diphtheria bacilli. These figures, unfortunately few in number, include

only *B. coli*, *B. tuberculosis*, and a strain of nitrobacteria. In comparison with these micro-organisms,⁵ the vibrios show a content of total mono-amino-acid nitrogen about 20 per cent higher than the other organisms, except the nitrobacteria, in which, however, the vibrio content is only approached. On the other hand, the total bases in the vibrios are markedly lower than those of diphtheria and *B. coli*, and about equal to those of the nitrobacteria and *B. tuberculosis*. Of these basic amino acids, arginine has about the same value in the vibrios, *B. tuberculosis* and the nitrobacteria, and is considerably lower than that of diphtheria. Histidine in the vibrios is about the same as in diphtheria, but has only half the value it has in *B. tuberculosis* and *B. coli*. The vibrio lysine value is similar to that in diphtheria and *B. coli*, and exceeds that of *B. tuberculosis*. Cystine is reported absent in our figures, as also in *B. coli*, nitrobacteria, and in some of the analyses made of *B. tuberculosis* and *B. diphtheriae*. It is probable that this failure to demonstrate cystine is due to a difficulty inherent in the method, rather than to a total absence of this important amino-acid.

The amide nitrogen of the vibrios is only about half of that reported in other micro-organisms, while the humin nitrogen occupies an intermediate position between that of the diphtheria bacilli (1 per cent to 2 per cent) and *B. coli* and *B. tuberculosis* (5 per cent to 6 per cent).

We may say in general that the vibrios investigated form a homogeneous group, well marked off in their nitrogen distribution from the other bacteria so far investigated. The vibrios are characterized in this investigation by having a relatively high content of the simpler amino-acids, while their basic amino-acid content is definitely low. The relative smallness of the amide nitrogen figure in the vibrios points to a comparative simplicity in their protein organization.

Since the van Slyke analyses reported here and the racemization studies to be reported later are different aspects of the same investigation, we will postpone discussion of these results until our subsequent paper.

SUMMARY

The protein fractions of six strains of agglutinating vibrios and two strains of non-agglutinating vibrios have been analysed by the van Slyke method. These proteins form a homogeneous group in respect to nitrogen distribution. They differ markedly, however, in nitrogen distribution from other organisms which have been similarly analysed. The evidence indicates that the vibrios have a relatively simple protein structure.

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CONTRIBUTIONS TO PROTOZOAL IMMUNITY

Part II

IMMUNITY TO MALARIA IN MONKEYS AND THE EFFECT OF SPLENECTOMY ON IT

BY

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INTRODUCTION

MANY investigators have recorded that man, bird and monkey, after their recovery from an attack of malaria, develop varying degrees of immunity to the disease, and that this immunity renders them partly or wholly refractory to subsequent inoculation of infective material* That such an immunity does develop in man has been demonstrated in individuals in malarious districts (*vide* Christophers, 1922, Thomson, 1924, James, 1931) In parietic patients receiving inoculations of malarial blood as treatment for their disease it has often been found impossible to produce a second attack by inoculation of infected blood (Yorke and Macfie, 1924) The results of experiments on avian malaria are also in general agreement with these findings and they, in addition, suggest that acquired immunity in birds is of the nature of an 'immunity to super-infection' (Moldovan, 1912, Whitmore, 1918, Manwell, 1930) Observations on monkey malaria indicate that acquired immunity does develop in these animals after recovery from one or more attacks

* A complete review of the subject has recently been published by MULLIGAN and SINTON (1933)

of malaria (Flu, 1918, Leger and Boulliez, 1913, Blanchard and Langeron, 1912, Taliaferro, 1932, Mulligan and Sinton, 1933). In the course of our cytological studies and splenectomy experiments in monkey malaria we made certain observations which corroborate the above findings and in addition stress the very important part played by the spleen and the reticulo-endothelial system in acquired immunity to malaria. We have therefore presented our observations and conclusions in this article.

MATERIAL

The monkey plasmodium used in our experiments was hitherto believed by us to be identical with *P. vivax*, but now Sinton and Mulligan (1933), from a detailed study of the parasite, consider it a new species and have named it *P. knowlesi*.

The immunity to infection with this plasmodium has been studied in three species of *Macacus* monkeys, namely, *M. rhesus*, *M. mus* and *M. radiatus*. The majority of the animals referred to in this article were previously used in cytological and other studies, observations relating to immunity made in the course of those studies are alone referred to here.

NATURAL IMMUNITY

That natural immunity, either partial or complete, to plasmodium infection does exist in monkeys is supported by the following observations —

The course of plasmodium infection varies greatly in different species of monkeys. In the *rhesus*, *P. knowlesi* causes an acute fatal infection, while in the *mus* and *radiatus* it produces a mild low grade infection which ends in spontaneous recovery.

When approximately the same dose of parasite is inoculated into a large number of monkeys of the same species, some of the animals fail to become infected. Two out of 27 *rhesus*, 2 out of 16 *mus* and 1 out of 5 *radiatus* could not be infected at the first inoculation. On repeating the dose again and again, 3 out of the 5 refractory animals became infected, one *mus* and one *radiatus* proving resistant till the end.

A study of the pre-patent and patent periods of infection, as well as the mortality rates in the treated and untreated monkeys (*vide* Krishnan, Smith and Lal, 1933), also shows that the degree of natural immunity varies in different species and in certain individuals of the same species.

ACQUIRED IMMUNITY

Fifteen monkeys, including 8 *rhesus*, 6 *mus* and 1 *radiatus*, received more than one inoculation of infective material after their recovery from an initial attack of malaria. The following is a brief summary of the history of the monkeys in general —

The primary acute attack of malaria which was generally treated with quinine lasted for a period varying from 4 to 10 days.

A short period (1 to 2 weeks) during which parasites were absent from the peripheral blood ensued, during this period no treatment was given.

Next a longer period (4 to 8 weeks) characterized by a varying number of relapses followed. The routine as regards treatment during this period was to give

quinine only when the number of parasites in the peripheral blood was over 5 per microscopic field

After this a very long period of freedom from infection of the peripheral blood resulted. This extended over several months and during this period the animals were apparently in normal health and no parasites were demonstrable in the peripheral blood. It was during this period that the animals showed varying degrees of acquired immunity. If inoculation of infective material was given during this period a high degree of acquired immunity was noticed, and even when it was repeated with massive doses it failed to produce infection.

In Table I results of re-inoculation have been summarized according to the species of monkey studied —

TABLE I

Showing the results of re-inoculation in the different species of monkeys

Results —	No infection	Transient infection	Severe infection	TOTALS
<i>M. rhesus</i>	4	3	1	8
<i>M. irus</i>	4	2	0	6
<i>M. radiatus</i>	1	0	0	1
Total number of animals	9 or 60 per cent	5 or 33·3 per cent	1 or 6·7 per cent	15

It will be seen from a perusal of the table that re-inoculation failed to produce infection in 9 out of 15 or 60 per cent, caused a mild transient infection in which parasites were present in the peripheral blood for a few days and then disappeared spontaneously in 5 out of 15 or 33·3 per cent, and gave rise to a heavy infection that had to be controlled by treatment in 1 out of 15 or 6·7 per cent. When these results are considered along with the clinical histories of these monkeys (omitted in the interests of economy in space) there is an indication—

- (1) that after one or more attacks of malaria, monkeys belonging to all the three species (*rhesus*, *irus* and *radiatus*) acquire a certain degree of immunity to re-inoculation,
- (2) that this immunity is more marked in the naturally resistant species than in the naturally susceptible species,
- (3) that the degree of acquired immunity increases directly in proportion to the duration of the primary infection and to the number of relapses, or re-inoculations that the animal gets after that,
- (4) that recovery from heavy infections does not appear to protect the animal more efficiently than recovery from milder infections,
- (5) that natural recovery from an infection confers better immunity than recovery following intensive treatment,

- (6) that infections allowed to persist in a latent form by judicious treatment confer better immunity than those that are rapidly overcome by excessive drugging, i.e., when the object of treatment is to check the severity of the symptoms only and obtain a cure through the natural immune processes of the body a far higher degree of acquired immunity is generally obtained

THE EFFECT OF SPLENECTOMY ON IMMUNITY

In a previous paper (*vide* Krishnan, Smith and Lal, 1933) we reported the effect of splenectomy on the course of malarial infection as studied in 30 monkeys belonging to three species. In that experiment we used 10 normal animals, 13 that had suffered previously from an attack of inoculation malaria and 7 that had definitely been proved to have developed a high degree of acquired immunity to re-inoculation. Extirpation of the spleen in these animals lowered the natural resistance of the first group and the acquired resistance of the second and third groups.

With a view to finding out if the resistance of monkeys to infection with the human plasmodium was also lowered by splenectomy we removed the spleens of two *M. rhesus* monkeys and then inoculated them with infected human blood from a case of benign tertian malaria. Neither of the two animals developed any infection even when the dose was thrice repeated.

IS ACQUIRED IMMUNITY AN 'INFECTION IMMUNITY'?

There has been a good deal of discussion as to the probable nature of acquired resistance in malaria. The prevailing opinion is in favour of the view that it is of the nature of an 'infection immunity', i.e., refractoriness to re-infection is dependent upon the presence of a 'sub-clinical' focus of residual infection in the host and when complete recovery takes place the animal loses its acquired immunity. In order to test the validity of this view it is necessary to have a reliable method of finding out the existence of a latent infection. Different workers have utilized different methods to accomplish this object. Some have injected the blood of the animal suspected to have a latent infection into a healthy one to see if infection would occur in the latter. Others have injected healthy blood (foreign protein) into the suspected animal so that it may help to convert the latent infection into an acute relapse. The results obtained by these methods show that they are very helpful but by no means reliable, because when negative results are obtained there is no certainty that the suspected animal is completely free from infection. From the results of splenectomy experiments we are led to believe that removal of the spleen may be a more certain method of demonstrating the presence of latent infections in experimental animals. By removing the spleen in 22 apparently healthy monkeys with a previous history of malaria we obtained an acute and severe relapse of infection in 14 of the animals and in the other 8 a marked susceptibility to re-inoculation was demonstrated. Had there been a residual infection in the latter group, it would have flared up after splenectomy, the presumption is that in the latter there was none. Therefore, so far as monkeys are concerned, splenectomy appeared to us to be a fairly accurate method for detecting latent infections. On

the basis of this assumption and with the object of finding out what percentage of monkeys, that had definitely been proved to have developed an acquired immunity to malaria were having a latent infection, we splenectomized 7 proved resistant monkeys and compared the relapse rate in these with those of other monkeys that had not been proved to possess any degree of acquired resistance. In Table II the results obtained are shown —

TABLE II

Relapse rate after splenectomy in monkeys that had previously recovered from malaria

Species of monkey —		<i>M. rhesus</i>		<i>M. mus</i>		<i>M. radiatus</i>	
		Relapsed	Not relapsed	Relapsed	Not relapsed	Relapsed	Not relapsed
Group I	Proved to be resistant to re-inoculation	3	2	0	1	0	1
Group II	Not proved to be resistant. Had recovered from an attack of malaria 6 to 18 months previously	6	0	5	3	0	1
TOTALS		9	2	5	4	0	2

Total number 22 Number relapsed 14 Number not relapsed 8

Every one of the 8 monkeys that did not relapse received a small dose of infective material and developed a severe infection. In the first group 4 out of the 7 monkeys that had been proved to have acquired resistance were found to have no latent infection. It is possible that in these the infection may have been lost during the interval between the date of re-inoculation and the date of splenectomy. It is therefore difficult to say whether at the time of re-inoculation there was any latent infection or not. On account of this and also because of the smallness of the number of animals experimented upon in each group no definite conclusions are justifiable, yet the following tentative conclusions may be drawn from the results obtained —

- (1) There is some evidence that a certain number of monkeys get rid of their malarial infection completely
- (2) In the susceptible species the number that does so is small while in the resistant species it is relatively large
- (3) Acquired resistance to re-infection is in a large number of cases dependent upon the presence of a latent infection

- (4) There is some evidence that in a small percentage of *rhesus* and in a larger percentage of *mus* and *radiatus* acquired immunity may be present even in the absence of a latent infection, there is, however, no evidence to show as to how long after complete recovery from infection acquired immunity generally lasts
- (5) Removal of the spleen results in a marked lowering of acquired resistance, in those in which immunity is due to a latent infection, severe relapse occurs and in the others in which immunity is present without any latent infection, a marked increase in susceptibility to re-infection results

Corroborative evidence of an indirect nature in support of the above conclusions may be obtained from a study of Table III wherein the number of days, during which latent infections persisted without any demonstrable sign in some of the splenectomized monkeys, is shown —

TABLE III

Showing the number of days during which the infection remained latent

Number of monkey —	1	2	3	4	5	6	7	8	9
<i>M rhesus</i>	121	127	245	290	299	456	466	467	476
<i>M mus</i>	40	206	208	244	245				

From the above table it will be clear that in the naturally susceptible *rhesus* the period of latency was much longer than in the *mus* monkey. Resistance to re-inoculation in both species of monkeys can be demonstrated even after periods of longer duration. So much so there is reason to presume that a few of the animals showing immunity might have had no latent infections.

THE RELATION OF IMMUNITY TO LATENCY AND RELAPSE

That the immunity of an animal may be an important factor in determining the onset of latency or relapse in malaria has long been recognized. These two conditions being the converse of one another, any factor such as an increase of immunity which favours the one will certainly hinder the other. Our cytological studies (Krishnan, Lal and Napier, 1933) indicated that latency generally follows a marked large mononuclear response which is a demonstrable sign of functional efficiency of the cellular mechanism of defence and our splenectomy experiments showed that relapse is the outcome of a depression in host immunity and a failure of the phagocytic mechanism. The observations recorded in this article stress the importance of the spleen and the reticulo-endothelial system in the causation of latency and relapse and in addition lend indirect support to the view of Ronald Ross, Bigami, James and Thomson, that during latency asexual multiplication goes on as usual and that the only difference between it and relapse is that a larger number of parasites get killed and fewer gain entry into red cells after each schizogony cycle.

A comparison of the number of days elapsing between splenectomy and onset of relapse in the animals harbouring a latent infection shows that it varied but slightly in the different species and was about 6 days, also the time taken to reach a height of infection of 1 million parasites per c mm of blood commencing from the first day of infection was also fairly constant in all the animals and was about 3 to 4 days. These are approximately the periods that would elapse in a very susceptible monkey after the inoculation of a small dose of infective material. Therefore the suggestion is that as a result of splenectomy there must have occurred a sudden failure of the immunity mechanism and a larger number of parasites must have survived after each schizogony cycle than previously, and brought on an intense relapse. During latency therefore one may presume that there is a sort of balance reached between host immunity on the one hand and the number of parasites on the other with the result that a more or less constant minimum of parasites survive after each schizogony cycle. Whenever one or the other of these two factors gain the upper hand, either recovery or relapse takes place. Thus, relapse is not a renewed activity of quiescent parasites but rather an expression of partial or complete failure of host immunity and of unchecked growth of the parasite population.

CHANGES IN THE SPLEEN OF IMMUNE ANIMALS AND THEIR SIGNIFICANCE

The splenectomy experiments provided us with a number of enlarged malarial and normal spleens and we made an attempt to study the relation of the size of spleen and the cytological changes present in it to immunity in malaria. In all 32 spleens from 10 normal and 22 malarial monkeys were studied and the results obtained are given below —

Spleen size — The malarial spleens were distinctly larger than the normal spleens and varied in size from 1 to 6 times the normal, the average being 3.6 times normal.

In 14 out of the 22 malarial spleens a latent infection was detected and in the other 8 there was no infection. The sizes of the spleens in the former group were distinctly larger than the sizes of the spleens in the latter group.

Seven spleens belonged to monkeys that were proved to be completely refractory to re-inoculation, 5 of these were from *rhesus* monkeys and one each from *mus* and *radiatus* monkeys. The two latter were of normal size but the 5 former were all enlarged.

When the size of malarial spleens of the different species of monkeys was compared it was found that the spleens from *rhesus* monkeys showed the greatest degree of enlargement, the spleens from *mus* a moderate degree of enlargement and those from *radiatus* only a slight enlargement. From these observations one may suggest that from the point of view of malarial immunity —

- (a) it is better to have a spleen even though it be enlarged, to get it removed is unwise,
- (b) to have had malaria is better than to have had none,
- (c) although acquired immunity is generally associated with enlargement of the spleen a spleen of normal size in the resistant species may be associated with a high degree of acquired immunity.

Cytological studies—From supravital examination of puncture material obtained from normal and malarial spleens the impression gained was that there was a distinct increase in the large mononuclear elements in the latter. A fair number of dividing monocytes were constantly met with in the malarial spleens. The number of dividing monocytes was greater in spleens that harboured a latent infection than in those that did not. Taking these findings in conjunction with the results of our previous cytological studies (Krishnan, Lal and Napier, 1933) we may state that there is some evidence—

- (a) that malarial immunity is closely associated with alterations in large mononuclear response,
- (b) that immunity is dependent not only upon the total amount of reticulo endothelial tissue present but also upon its functional efficiency and its sensitiveness to respond to the stimulus of infection,
- (c) that in the majority of resistant animals the sensitiveness of the reticulo endothelial system is kept up by the presence of a well-controlled low-grade infection,
- (d) that a demonstrable sign of acquired immunity is the presence of a fair number of dividing monocytes

Pigment—In malarial spleens harbouring a latent infection the pigment was fresher and larger in amount and less dark than in those that were free from infection

AN ECONOMICAL METHOD OF MAINTAINING THE STRAIN OF MONKEY PLASMODIUM IN THE LABORATORY

Workers have found the maintenance of plasmodium strains in monkeys a difficult and costly affair, for, either they cause acute and rapidly fatal infections in the susceptible species like the *rhesus* or a mild low grade infection which is rapidly overcome in the more resistant species like the *mus* and *radiatus*. If the first species of monkey is used rapid transfer from one animal to another has to be performed and this would entail a tremendous cost, on the other hand if it is proposed to maintain the strain in the resistant species then there is no knowing as to when the infection will be completely overcome and the strain lost. From the results of our studies on splenectomy in monkeys recorded in this article we are led to believe that an economical method of maintaining the plasmodium strain in the laboratory over long periods during which no experimental work is contemplated or for transporting infected animals from one laboratory to another will be as follows —

A few healthy *rhesus* monkeys are chosen and inoculated with the plasmodium. As soon as infection is established the animals are treated with minimal doses of quinine till parasites disappear from their peripheral circulation. Watch is kept for about a month on the animals by repeated blood examinations for any relapses of infection and, when they occur, treatment is given, only to rid the peripheral blood of parasites. After this the animals can generally be kept in the laboratory without any special attention being paid to them. Periodically their blood may be examined if desired, but this we have not found necessary. We have kept them

for periods of a year and more in this way. Whenever there is a demand for infective material splenectomy is done on one of the animals and within a week an intense infection results which will furnish sufficient material for any purpose.

SUMMARY AND CONCLUSIONS

(1) There is evidence that a certain degree of natural and acquired immunity to malaria does exist in monkeys.

(2) This immunity is markedly lowered by the removal of the spleen.

(3) Although acquired immunity in the majority of instances is due to a latent infection, there is some indication that in a small percentage it may be present in the absence of any such infection. This particularly applies to the *irus* and *radiatus* monkeys.

(4) In the susceptible species increase of immunity is generally associated with enlargement of spleen, but in the resistant species immunity may be present even when the spleen is of normal size.

(5) The presence of dividing monocytes appears to be an indication of the sensitiveness of the reticulo-endothelial cells and of the presence of a latent infection and immunity in malaria.

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THE DISTRIBUTION OF GOITRE IN THE UNITED PROVINCES *

Part IV

FURTHER NOTES ON THE ÆTIOLOGY OF GOITRE IN THE SUPER-ENDEMIC AREAS OF THE GONDA AND GORAKHPUR DISTRICTS

BY

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A GENERAL ÆTIOLOGICAL FACTORS

1 *Age* —Goitre is rare below the age of three years. Between 3 and 5 years the rate in the two super-endemic areas visited is less than 5 per cent, though this percentage rises higher in severely affected areas. Children between 6 and 14 years were examined in many schools and the incidence was 10 to 20 per cent, but the rate may reach 80 per cent in areas of greatest endemicity. Between 15 and 30 years the rate rose to 60 per cent and fell again above 30 years. In the most severely affected areas of Padrauna the rate was about 90 per cent above the age of 5 years.

* During 1932 Dr S P Gupta, M D (Lucknow), toured the two super endemic goitre areas of Gonda and Gorakhpur and submitted notes which are set out under sub heads similar to those in Parts I, II and III of this work which appeared in the *Indian Journal of Medical Research* for April 1931 and July 1932. The notes are supplementary to that information which they extend —[H S]

2 *Education and wealth*—Very few educated and well-to-do persons acquire goitre even in the worst endemic areas. Educated persons are generally in Government service and are mostly immigrants from non-goitrous areas. They keep their private wells in good condition, regularly cleaning and occasionally permanganating them. Officials, especially Europeans, drink boiled water. Almost all the well-to-do, including the zemindars, have married in non-goitrous areas or to non-goitrous families. Once the disease starts treatment alone without anti-goitre precautions is insufficient to check its development. Many families do not regard the slight neck swelling due to early thyroid enlargement as 'goitre', and only recognize it as such when the goitre is of large size, which is usually first in the females. Nor do such families persist in treatment, for they do not attach much importance to the goitre.

3 *Sex*—Goitre is definitely more prevalent in females and in some such proportion as 3 females to 1 male. In any family if one male had goitre probably not a single female of that family was goitre-free, except perhaps the very old ones. In certain villages females without goitre are rare.

4 *Family*—Goitre is certainly a family disease. This was most obvious in the less affected areas where goitre is often confined to certain families only in the village. Inheritance is an important factor not only in the production of deaf-mutes and cretins but also in increasing the tendency to the production of goitre. But children of goitrous parents born in or immediately removed to non-goitrous areas do not often develop goitre. Race and community are not connected with the incidence of goitre.

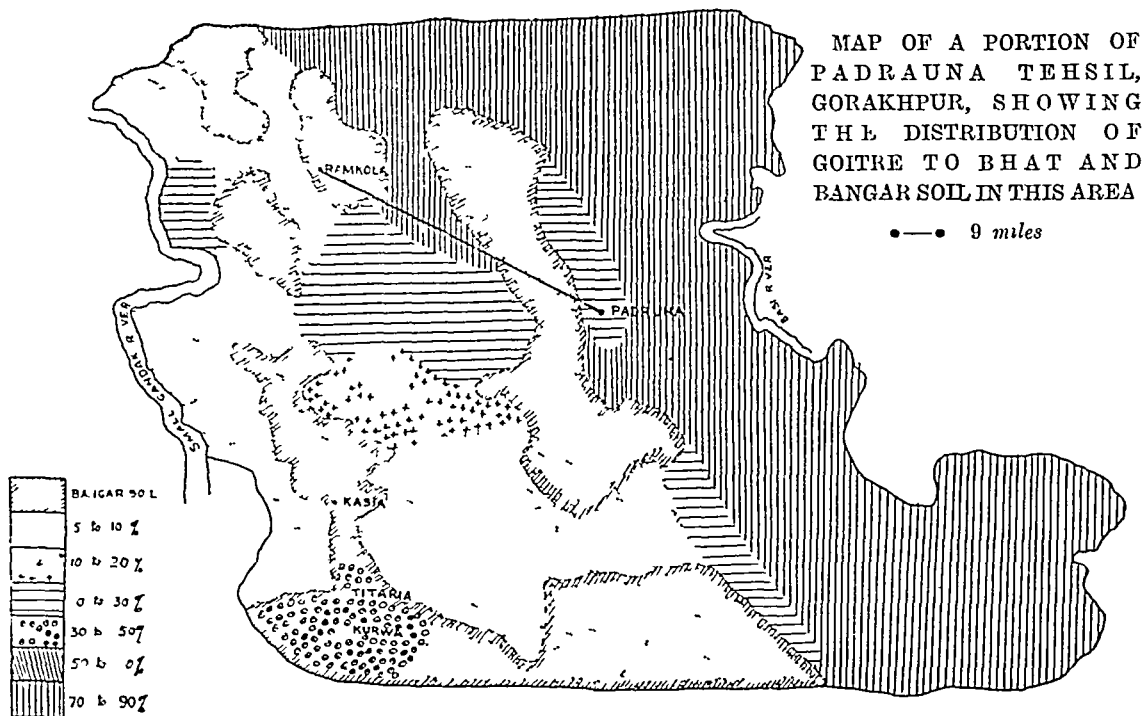
5 *Goitre in animals*—Dogs affected with goitre are commonly found in the endemic areas. A newly born kid was observed with goitre. A buffalo and a cow were brought with supposed goitre but the tumour was not goitrous but probably lipomatous. Cats and birds are said to be affected. Jackals with goitre were not reported.

6 *Season*—New goitres arise and old goitres enlarge in the winter season. The increased attendance of goitre out-patients in winter is not due so much to an increase in the goitre rate as to the fact that blisters caused by the red iodide of mercury ointment universally prescribed are far less painful in winter months than in summer. In the rains the flooded areas also greatly diminish the out-patient attendances.

7 *Associated diseases*—*Malaria* is quite common on account of the floods. *Round-worm infection* is so common that every child with gastro-intestinal troubles is treated for this condition. About 10 per cent of the school children had passed round-worms. *Hydrocele* is common in some localized areas and is now said to be definitely increasing. Several cases of hydrocele with goitre were reported. Hydrocele does occur both in goitrous families and villages. A few cases of leg *elephantiasis* were seen. *Cholera* is endemic in both areas for more than 6 months in the year. *Plague* is common but it has never affected the area within ten miles radius of Padrauna proper.

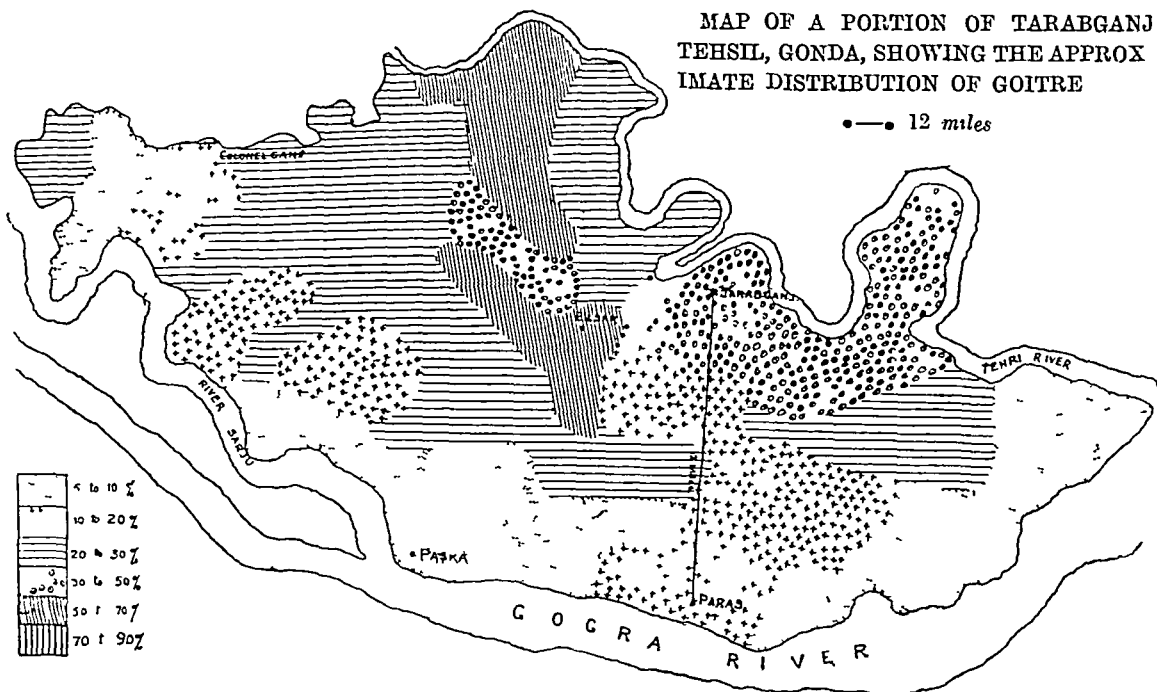
8 *Increase and decrease of endemicity in the two super-endemic areas*—It is locally believed that goitre is decreasing in the Padrauna Tehsil (Gorakhpur) but is increasing in Tarabganj Tehsil (Gonda). Some 100 years back the Padrauna endemic area, including the town of Padrauna, was even more severely affected.

MAP 1



The soil elsewhere than shown as Bangar soil, so far as the white appearance goes, is Bhat soil

MAP 2



with goitre than it is at present as judged by (1) numerous goitres of large size, (2) many deaf-mutes and cretins, and (3) goitres in cats, dogs, birds and other animals. The severity of the goitrous influences has certainly decreased in the sense that large goitres, deaf-mutes, cretins and goitrous animals are far more rarely seen, but, including such small goitres as are indicated by an appreciable neck swelling, the goitre rate in that part of the tehsil north and south-east of the teshil of Padiuna still reaches 90 per cent. The decrease is much more noticeable in Padrauna town. The decrease is partly due to the influx of persons from non goitrous areas into the town for trade purposes, who have not developed goitre because of the weakened goitrous influences. In both Padrauna and Tarabganj the goitre rate diminishes southwards. The moist area of the Tarabganj Tehsil is along and between the rivers Chandha and Tehri. The strip of land along the north bank of the Sarju and Gogra rivers is not completely free and at places the rate approaches 10 per cent. The two maps attached show the distribution of goitre in the areas surveyed.

B SPECIAL ETIOLOGICAL FACTORS

The information collected in this recent survey of these two areas and reproduced below should be read as supplementary to that already set out under soil, water-supply, diet and salt (Stott *et al*, 1931).

1 *Diet and salt*—In Gonda, the poor labourer generally consumes Ganji (sweet potato) and Junhri (maize) in winter and Jowar and Bajra (small grains) in summer. Many people believe that goitre is due to 'Ganji', but there is no Ganji produced in the western part of Gonda or in Padrauna where rice and maize form the main diet. Diet does not appear to play a part in the production of goitre, for many poor people are not affected though they are taking the same diet as persons with goitre while some of the well-to-do who live on good quality food are affected. About 15 years ago some American missionaries and their children developed goitre at Padrauna.

Salt—In both areas Sambhar (sea-salt), not rock salt, was used.

2 *Soil*—In the Padrauna Tehsil the soil is of two types. (i) *Bangar* which covers about 25 per cent of the tehsil and is dirty yellowish in colour from its clay content, hard to plough, and requires much irrigation but yields a produce of a superior quality.

(ii) *Bhat* is of two types—

(a) *Pure Bhat* soil is dirty white in colour, soft, friable and amorphous in consistency and has great capacity in retaining moisture. Irrigation is therefore not required. The produce is of medium quality.

(b) *Sandy Bhat* (Dhusa) is of sandy colour. The produce is of the lowest quality, being, as people say, just sufficient to pay off the dues.

Broadly speaking, the distribution of goitre in Padrauna Tehsil corresponds with the distribution of Bhat and Bangar soils. Super-endemic areas are limited to Bhat soil only. There are, however, villages on Bhat soil with a goitre rate under 5 per cent. Such villages are Sisiva, Narkatia, Chaupana, Ahiraul, Rampur, Mathianwan, Karhaya, Ghur Chapra, and Kissmha. Padrauna Chaoni is situated half on Bhat soil and half on Bangar. That portion on Bhat soil shows

many goitre cases, while the other half of Bangar soil is practically free. The well in the affected portion which has existed for many years is stated to have become goitrous only within recent years. In the village Domar Bhar situated on Bhat soil goitre is limited to one section only though both sections are only two furlongs apart. On the other hand, some villages on Bangar soil have a goitre rate of about 30 to 35 per cent, e.g., Titaria, Kurwa and Dileepnagar. The subsoil water is high. Many wells in Bangar soil and a few in Bhat are reported to possess the property of curing goitre. Though Gonda alluvium also contains much calcium, there is not the same sharp distinction between two types of soil in Gonda—as in this Padrauna area.

3 WATER-SUPPLY

There is a considerable volume of evidence that the cause of goitre is water-borne. The villagers in the main believe that goitre is water-borne and not diet-borne. Hence the relation of drinking water to goitre cases, families and localities merits detailed attention.

(1) *Shallow wells easily polluted*—In both the super-endemic areas of Tarabganj and of Padrauna, the water-supply is from wells, even in villages on the banks of Sarju. These wells are shallow, uncovered, unprotected by a parapet wall, close to dwelling houses and to dirty ponds and are unsupervised. The water is often at the same level as the surrounding ponds. Household utensils are freely dipped in the well, and during rain and flood period surface washings readily pass into the water. The chance of contamination is therefore very great.

(2) *Goitre wells*—Many wells are recognized as 'goitre' wells. A well of Bansī village in Padrauna which was dug 20 years back but was subsequently closed as it was found to produce large goitres. Some wells are said to have become goitrous only recently, e.g., one in Changeri village, Gonda district, only during the last 7 years. Goitre wells, like non-goitre wells, frequently contain 'hard' water. Milkiness in either kind is rare. Some goitre wells are 'only'. In some villages when building new wells, no goitrous person is allowed to approach as it is believed that should such a man approach the well will become goitrous. There were families amongst whom there had been no goitre until some goitrous individual (e.g., a bride) joined the family, after which goitre appeared amongst several members of the family.

(3) *Contamination of water by remains of bygone jungles in alluvial deposits*—The priest at the Buddhist shrine of Matha-kunwar and the Raja Bahadur of Padrauna state that hundreds of years back most of the area of the Padrauna Tehsil was the bed of Great Gandak river. Later, the bed of this river was filled in by soil brought down by the Gandak river and its tributaries, and later still this soil became covered by jungle. In a similar manner, the endemic area in Tarabganj Tehsil is located on what was once the bed of river Sarju. There also the jungle period followed. Every year a layer of dead leaves and plants was covered with a layer of fresh alluvium, carried down by the rivers during the floods. Thus, vegetation remnants and roots and mud and perhaps calcium formed the first soil on which man began to establish himself after cutting away the jungle. As the trees were felled the buried organic matter which was formerly utilized by those trees began to rot.

(4) *Organic and bacterial contamination of water-supply*—In Padrauna, but not in Tarabganj, many of the shallow wells are of a dirty greenish colour, and on standing an oily film-like kerosene forms on the surface of the water, which has also the following characteristics —

- (1) the thick oily surface film, mentioned,
- (2) a bad smell from decomposition,
- (3) rotting organic substance, e g , a coal-like material was recovered from a well at Dileepnagai in Bangar soil,
- (4) an astringent taste from organic acids,
- (5) 'browning' of brass lotas by organic acids,
- (6) 'hardness' due to solution of lime from the soil by the carbon dioxide set free from decomposing organic material,
- (7) even a muddy appearance from suspended organic matter which would deposit on standing

Such evidence of gross pollution is supported by the large amount of permanganate of potassium required to 'pink' it, the yearly cholera epidemics and the high local worm infection rate

The high subsoil water-level, the dropping of rotting leaves, vegetation and surface washings into the water provide an excellent culture medium for germ growth, which gain easy access from human and animal excreta and other sources. But vast numbers of Indian villages away from the Himalayas also drink water from such polluted wells without developing goitre. The wells of villages remote from the Himalayas do not contain that special soil of decade-old organic material derived from the rotting of bygone jungles at the foot of the huge mountain ranges like the Himalayas and containing the deposits brought down from limestone rocks by such rivers and referred to under the previous heading. The oily film was much more marked in the severely affected Padrauna area, and was not observed in Tarabganj.

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THE DISTRIBUTION OF GOITRE IN THE UNITED PROVINCES

Part V.

FURTHER CLINICAL NOTES ON ENDEMIC GOITRE, SUB-THYROIDISM CRETINISM AND DEAF-MUTISM IN SUPER-ENDEMIC AREAS OF GONDA AND GORAKHPUR DISTRICTS

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1 CLINICAL OBSERVATIONS

A The neck swelling and its local changes

1 *The goitre incubation period* — When a person is continuously exposed to the goitre-producing influences of an endemic area a swelling of the thyroid gland frequently develops. The time required before the goitre becomes apparent (i e , the goitre incubation period) depends on the five factors mentioned below. The rate of increase in size of the goitre varies with the same factors —

(1) *Age* — Children of about 3 require several years' exposure. Children of about 10 years, whether male or female, require 6 to 12 months' exposure. Adolescents are most readily affected and that after exposure for only some 3 to 6 months. After adolescence as age increases, the period of exposure required increases. Above 45 years goitre will probably not develop at all. Persons therefore are most susceptible about puberty.

(2) *Heredity* — A goitrous heredity decreases the incubation period

(iii) *Sex*—The incubation period is shorter in females than in males

(iv) *Season*—Persons who settle in the goitrous area just before the rains may often develop goitre during the following cold weather, whilst persons who settle in the cold season may not show any signs until the next cold season

(v) *The intensity of the endemic goitrous influences*—Naturally the greater these influences are, the shorter will the incubation period tend to be

2 *The early signs and natural history of the enlargement*—In some 98 per cent of cases, the goitre first appears as a diffuse fullness of the space above the jugular notch and a lessening of the prominence of the insertions of the sternomastoid muscles bounding it. In a few cases the enlargement starts as a nodule in the isthmus or in the right thyroid lobe. The left lobe appears rarely first affected. Under continued goitrous influences the swelling increases as a diffuse or nodular enlargement which finally becomes stationary or again starts to enlarge after an interval of several years. A common stationary size is that of a cricket ball, but in the Gonda district there are many cases where the goitre had reached the size of a football. In old age the size is apt to lessen from fibrotic changes. Even the diffuse type assumes a nodular appearance in old age.

3 *Pressure symptoms*—Pressure symptoms are frequent, and do not depend on the external size of the goitre. Many persons with large external goitres suffered no difficulties, whilst those with goitres which appeared small showed pressure symptoms. Dyspnoea and dysphagia were the only complaints made. One goitrous subject required two hours for his meal. In others, a constant respiratory wheeze was present. No case of pressure on the recurrent laryngeal nerve with hoarse speech was observed. A few cases showed distended prominent veins over the surface of the goitre with congestion of the face and eyes from venous obstruction.

4 *Suppuration and malignancy*—Two cases were seen with chronic neck sinuses connected with a slightly enlarged goitre. These cases stated that formerly they had large goitres which had suppurated but the sinuses were not healing. No cases of malignant goitre were seen in this survey.

5 *Treatment of goitre*—The only treatment available in villages is the red iodide of mercury ointment which blisters the skin and acts as a counter irritant. Indigenous practitioners (*Vayds*) also apply certain herbs which raise blisters. Tincture of iodine is sold as a local application for the cure of goitre. Both applications appear to cure early goitres and reduce the size of big ones. Iodine is also taken internally and is being tried in the Gonda schools as a prophylactic measure. Complete cure is rare. It appears probable that after the iodine treatment is stopped the patient will again develop goitre unless the goitre-producing influences are removed.

B The endocrine disturbance with physical and mental changes

1 *Sub-thyroidism*—A goitre itself may have little appreciable effect on physical and mental development, especially when acquired by new-comers to the



Fig 1 Big goitre with dilated veins on goitre and chest



Fig 2 Big goitre with pressure symptoms (One hour to feed)



Fig 3 Deaf mute Fascies with goitre



Fig 4 Cretinous deaf mute Infantile sex organs Age 16

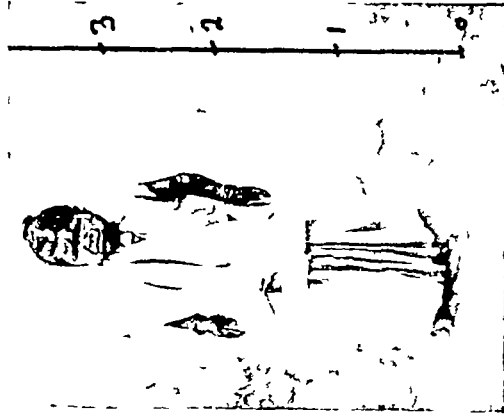


Fig 5 Cretin of 16 Sensible



Fig 6 A cretinous deaf mute of 20 with a normal man of same age

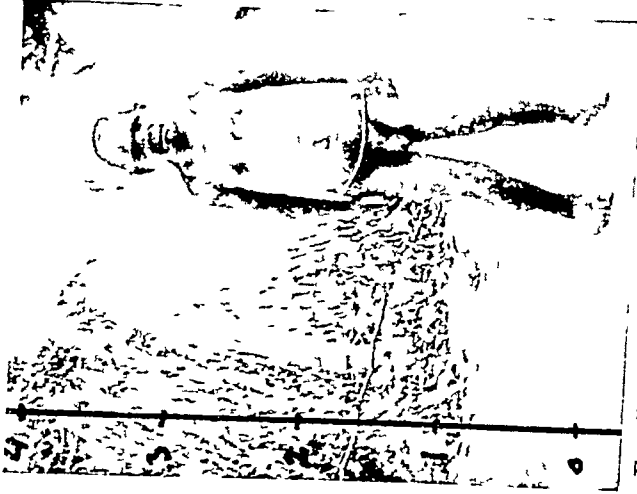


Fig 7 A cretin of 20 Able to speak



Fig 10 A cretin of 10 Sensible

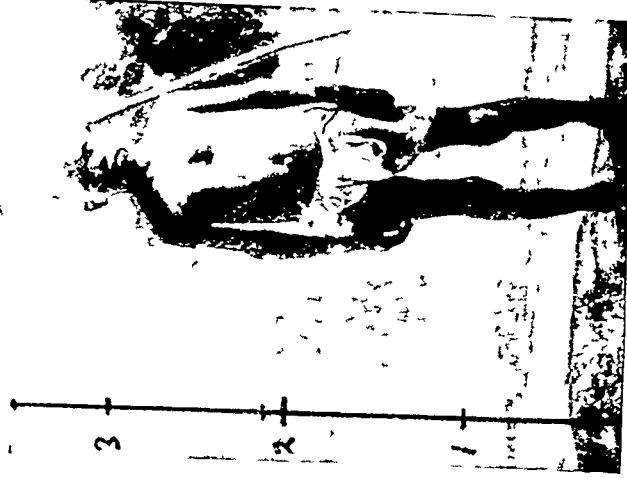


Fig 8 A cretin of 25 Deaf mute

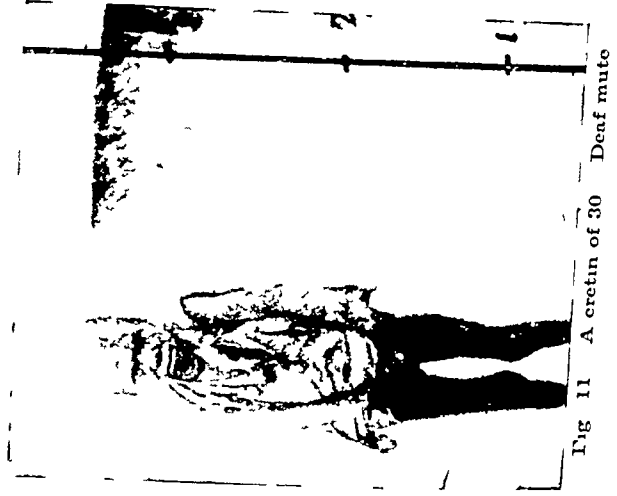


Fig 11 A cretin of 30 Deaf mute



Fig 9 A cretin of 25 Sensible

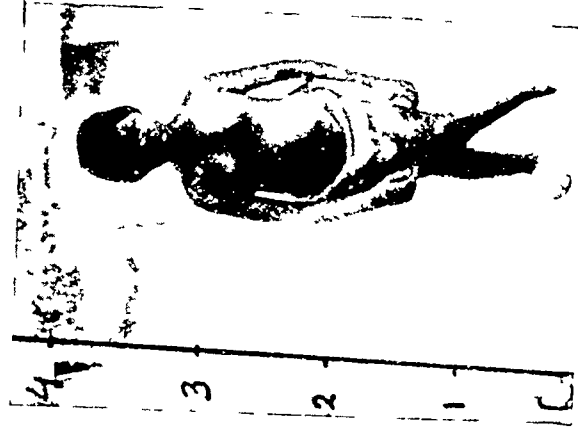


Fig 12 A cretin of 30 Deaf mute Showing the knock knee

locality. No signs of *hyper-thyroidism* were seen amongst many thousands of persons observed in these super-endemic areas. On the other hand many cases of *sub-thyroidism* were noted as the endemic villages were traversed. In bad areas the mentality was low. School teachers complain that an average student knew much less and required more teaching than one in a non-goitrous area. Evidence of *myxœdema* was noted in different cases as under (1) a thick dry scaly skin, (2) thick, puffy, pale faces with narrow palpebral fissures showing half-closed eyes and with thick lips and a thick tongue, (3) hair deficiency of the face armpits and pubis, (4) lowered body metabolism, sluggish movements and slow circulation. Such signs of *sub-thyroidism* were mostly observed in congenital cases, though some older persons had acquired this condition during a life-time passed in the worst endemic areas, and (5) slow cerebration slow speech and hearing—all greatly intensified by cold. But advanced cases showing the complete syndrome were rarely seen.

2 *Cretins*—The following observations on 35 *cretins* were recorded—

(a) *Parents*—*Cretins* are born to goitrous parents who did not themselves necessarily show signs of *myxœdema*. *Cretins* were not born unless the family had resided for at least 3 or 4 generations in the endemic area.

(b) *Goitre rate* in these 35 *cretins* was about 60 per cent.

(c) *Age distribution*—10 to 20 years, 12 (34 per cent), 20 to 30 years, 12 (34 per cent), 30 to 40 years, 7 (20 per cent), over 40 years, 4 (12 per cent), total 35.

(d) *Sex* of the 35 seen, only 7 were females.

(e) *Height*—All were dwarfs ranging between 3 feet 5 inches and a few inches over 4 feet at adult age.

(f) *Body condition*—56 per cent were fat, 24 per cent of medium build, and 20 per cent were thin.

(g) *Framework and features*—The large head, massive trunk, and small extremities with coarse thickening of the subcutaneous skin, eyelids, lips and tongue, with small slit-like palpebral fissures, depressed nose-bridge and scanty hair growth were typical. The teeth were retained. The sex organs were usually normal except in the severe cases. Very few were married. Only two had children. The severe cases were pale and anæmic.

(h) *Mental capacity*—Three might be classed as true *cretins* and 32 as *cretinoids* or semi-*cretins*. In some the mental capacity was only slightly subnormal and such could carry on shop or field work but were easily irritated. One would not stand before the camera though his friends explained how harmless it was. Some presented definite signs of *myxœdema* and in these the sluggish mental picture was especially marked. One appeared quite disinterested in his surroundings but smiled when it was suggested that his photograph might be used to arrange for his marriage.

(i) *Speech and hearing*—Speech was usually slow and defective, some could only speak after a long latent period. Most had defective hearing. About 50 per cent of the *cretins* seen were deaf-mutes.

3 *Deaf-mutes*—The following observations on deaf-mutes are recorded. In the areas of super-endemicity visited the deaf-mute rate worked out at 230 per 100,000 of population or 2.3 per 1,000. Only congenital deaf-mutes were reviewed. Such are sometimes described as cases of endemic deafness or cretinic deafness.

(a) *Parents*—Deaf-mutes are frequently born to goitrous parents in endemic areas. In 80 per cent of deaf-mutes, both parents had goitre, in 15 per cent only the mother and in 5 per cent only the father had a goitre.

(b) *Goitre rate amongst these deaf-mutes* was 90 per cent, which was mostly not acquired in childhood but developed as they grew older.

(c) *Deaf-mute rate amongst the cretins*—50 per cent of 35 observed cretins were deaf-mutes.

(d) *Myxœdema rate*—Many deaf-mutes showed some signs of myxœdema.

(e) *Relation to goitre areas*—Deaf-mutes are commonest in areas of highest endemicity. The distribution of deaf-mutes varies directly with the distribution and with the intensity of goitre. But the deaf-mute rate increases later and recedes early in the history of the rise and fall in the goitre endemicity of an area.

(f) *Age distribution*—

Years	Gonda	Padrauna	Average (percentage)
0-10	20	22	21
10-20	65	46	56
20-30	9	21	15
40-50	5	10	7
Over 50	1	1	1
TOTALS	100	100	100

Taking the deaf-mute rate as a measure of the goitre intensity, it would appear that goitrous influences were more active some 20 years back than they are at present.

(g) *Sex*—About 70 per cent of the deaf-mutes seen were males.

(h) *Characteristic gait and appearance*—Deaf-mutes can usually be recognized at a distance by their characteristic gait. Knock-knee is usually present and the feet usually turned in. The heels are slightly raised and point outwards. The knees, and sometimes the hips, are slightly flexed. Progression is by short quick

paces, at almost an 'amble' with the arms held somewhat stiffly vertically downwards. Signs of sub-thyroidism are generally present. The expression is idiotic, with thickened lips and tongue, dirty teeth and a depressed nose-bridge. The lower lip is often slightly everted and saliva may dribble out.

(1) *Height* — Except those showing cretinism the height was the normal for the area.

(2) *Mental capacity* — Even cretinous deaf-mutes usually know how to satisfy their wants and understand gestures made to them. Their work was mostly limited to guarding herds in the jungle, but some of them are able to carry out field or even house work. A small percentage are so lethargic as to be useless to the family. A traveller in these areas will often call to a field labourer asking for directions as to his way only to find that he is uselessly addressing a deaf-mute. The cause of congenital endemic or cretinous deaf-mutism has been shown microscopically to be due to myxomatous thickening of the submucous tissues of the middle ear. Professor Nager of Zurich believes that the condition arises about the sixth month of foetal life. Tuning-fork tests show that many cretinous deaf-mutes are really only 'hard of hearing' but that this degree of deafness, super-added to a feeble mental development, results in deaf-mutism.

SUMMARY

Clinical observations of the neck swelling and of its incubation are recorded. Pressure symptoms were often observed. Sub-thyroidism was common. Thirty-five cretins were analysed. Many were photographed. A deaf-mute gait is described and observations on deaf-mutes are recorded.

IDENTIFICATION OF LARVÆ OF THE GENUS *PHLEBOTOMUS*

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THE fruitful researches that have been conducted within the last two decades or more have already established the importance of sandflies (*Phlebotomus*) as carriers of disease. Members of this genus are suspected of transmitting various diseases, and though it has not been possible to obtain definite proof in all instances, in two at least this has been achieved. The work of the Indian Kala-azar Commission and the ancillary Kala-azar Inquiry on the one hand, (*vide* Shortt, Smith, Swaminath and Krishnan, 1931, Napier, Smith and Krishnan, 1933) and the researches of Doerr, Franz and Taussig on the other, have clearly established the fact that *P. argentipes* is a vector of kala-azar and *P. papatasi* of sandfly fever. These two diseases have a wide distribution and are the cause of a large amount of morbidity and mortality in the regions where they occur, and in consequence the sandfly has come to occupy a place in the long list of arthropod carriers of disease which is of no less importance than that of the mosquito, muscid, tick or flea. In order therefore to help in the institution of appropriate control measures against *P. argentipes* and to facilitate the methods of prevention to be adopted against kala-azar, we have undertaken a study of the bionomics of these flies and have in this paper presented the differential characters of the larval forms of the three common species found in Bengal, viz, *P. argentipes*, *P. papatasi* and '*P. minutus*'*.

Larvæ of *Phlebotomus papatasi* have been described by Newstead (1911) and Whittingham and Rook (1923). Howlett (1915) studied the very early

* Used as a general term in this paper to denote an unidentified species of this group

stages of the three species under consideration—*P. argentipes*, *P. papatasi* and '*P. minutus*' and described a method of differentiating them. In his paper he used the length and relative thickness of the caudal bristles of the larvæ as diagnostic points. For our purpose and for the correct identification of these larvæ generally we have found that these points are not by themselves of sufficient practical value. The larvæ, which are obtained from the breeding grounds investigated by us, are more often than not full-grown ones, the method used in collecting these larvæ, by repeated washing of the sample of earth and their passage through sieves of various sized mesh, very often results in the hairs of the larvæ being broken or rubbed away, and for this reason differentiating points, other than those described by Howlett, are often required.

For the purpose of our present study we bred *P. argentipes*, *P. papatasi* and one species of the '*P. minutus*' group in the laboratory and observed the characteristics in numerous larvæ, before we finally decided on any point of differentiation. We have found that there are differences not only in the character of the hairy spines with which these larvæ are covered, but also in the arrangement of these spines on the head and body of the larva. We have only used one species of '*P. minutus*' (*sensu lato*) for the purpose of this paper, to show the difference between the *P. minutus* group generally and *P. argentipes* and *P. papatasi*. The most important species in Bengal is *P. argentipes*, the carrier of kala-azar, a correct identification of the larvæ of this species is necessary if the breeding grounds of *P. argentipes* have to be sought for and rendered unsuitable for its breeding.

TECHNIQUE

The larvæ were first washed in water to get rid of any adherent particles of debris and then transferred to pure carbolic acid, and, as soon as the larvæ were seen to be quite cleared, they were dropped into a solution consisting of one part of clove oil to five parts of acid carbolic.

The larvæ were then mounted in this fluid in hollow ground slides and the coverslip sealed on with Apathy's cement (equal parts of hard white paraffin and Canada balsam). The figures appended were all drawn with the aid of camera lucida.

DIFFERENTIAL CHARACTERS

The larvæ of *Phlebotomus* which are composed of a head and twelve segments have two pairs of caudal bristles which are characteristic of the genus. The head and body of the larva are sparsely clothed with hairy spines. The body segments may be divided into three parts—the anterior or thoracic part consisting of three segments, a middle or abdominal part and a posterior part including the ultimate and penultimate segments—the distribution and character of hairy spines in these three regions being different in the different species. The terminal segment carries the two pairs of caudal bristles which are distinctive of *Phlebotomus* larvæ.

In full-grown larvæ there are certain differences noted in the area of chitinization of the dorsal surface of the ultimate segment (Mukerji, 1931).

In '*P. minutus*' about two-thirds of the segment are chitinized and anterior limit of chitinization has a concave border.

In *P. argentipes* the area of chitinization is not much larger but the anterior limit of the chitinization has a convex border

In *P. papatasu* practically the whole segment is chitinized and the chitinization borders on the lateral surface as well

The hairy spines—The hairy spines with which *Phlebotomus* larvæ are clothed show certain differences in the different species

The most distinctive are those of *P. papatasu* which have a transparent ovoid mass on the head of each spine. This character is not seen on the spines of the other two species. The hairs on the spines too seem shorter and closer to the main stem than in *P. argentipes* and '*P. minutus*'

Between *P. argentipes* and '*P. minutus*' the difference in the spines is not so marked but, observed with a low power, the spines on *P. argentipes* seem to be clothed with smaller hairs from the base upwards while in '*P. minutus*' the smaller hairs begin from above the base. In one species of '*P. minutus*', found in the washing of a sample of earth and not yet identified this difference is much more marked—the main stem of the spine being practically unclothed with hairs for a certain distance from the base, and the spine resembles a miniature feather duster

The head spines—In Plate XXXVII, figs 1, 2 and 3, will be found drawings of the larval heads of *P. papatasu*, *P. argentipes* and '*P. minutus*'. It will at once be seen that there are eight hairy spines on the vertex of the head of each larva. To localize these hairy spines use is made of the Y-shaped suture on the larval head which is a prominent feature—the lower limb of the Y reaching the posterior border of the head. The hairs just within the two limbs of the Y are referred to as the posterior hairs, *vide* Plate XXXVII fig 1 (IV), the two in front of these as the anterior hairs, *vide* Plate XXXVII, fig 1 (I), the other four hairs are referred to as postero-lateral (III) and antero-lateral (II) according to their position. We are not using the bristles for purposes of identification in this paper

It will at once be apparent from a glance at the figures that the two anterior hairs of '*P. minutus*' are different from the others on its head as well as from those of *P. argentipes* and *P. papatasu*. These hairs are very slightly branched, are slender and elongated

In all three species the position of the anterior and antero-lateral and posterior hairs does not seem to vary much at all. The differences noted are mainly due to the alteration in position of the postero-lateral hairs

In '*P. minutus*' the other six hairs are disposed of in a semi-circle on the vertex of the head with the convexity of the arc towards the base of the head, *vide* Plate XXXVII, fig 3

In *P. argentipes* the postero-lateral hairy spines are placed distinctly behind the posterior hairy spines, *vide* Plate XXXVII, fig 2

In *P. papatasu* the postero-lateral hairs are almost in a line with the posterior hairs, *vide* Plate XXXVII, fig 1

Body of larva. Dorsal surface—Examination of the dorsal surface shows that each segment has a transverse row of six hairy spines, three on either side of the middle line. In *P. argentipes* and *P. papatasu*, *vide* Plate XXXVII, figs 5 and 4, in

addition to these rows of spines there are short spines placed in between the rows of transverse spines on the abdominal segments of the larva. Seven such pairs are seen. In '*P. minutus*', vide Plate XXXVII, fig 6, these hairs are so minute that they cannot be seen except with the high magnification. Also, in '*P. minutus*' there is a difference noted in the middle pairs of transverse row of hairy spines—these hairy spines are very minute on the penultimate segment and also on the two segments preceding it—these hairy spines then show gradual enlargement till in the thoracic segments they are of nearly the same size as the other spines.

Lateral surface—The hairy spines on the lateral surfaces vary in the three species.

In *P. argentipes* there is but one hairy spine on each abdominal segment, vide Plate XXXVIII, fig 1. In the thoracic segments there are three hairy spines, vide Plate XXXVIII, fig 5, two being large and one minute. The larger spines are referred to as the dorso-lateral and ventro-lateral according to their position. The minute hairy spine is situated at the base of each dorso-lateral hair on the 2nd and 3rd thoracic segments.

On the 1st thoracic segment the hairs are differently arranged—a minute hair is placed ventral to the dorso-lateral hair, and there is a small hairy spine placed between the spiracle and the dorso-lateral hair.

In '*P. minutus*', vide Plate XXXVIII, fig 3, there are two lateral hairy spines on each abdominal segment. In the thoracic segments, vide Plate XXXVIII, fig 4, there are three—two large and one smaller hairy spine, the smaller spine being at the base of the dorso-lateral spine and being much larger than the corresponding spine on *P. argentipes*. On the 1st segment in '*P. minutus*' this hairy spine is not at the base of the dorso-lateral hair but ventral to the spine in question, in much the same position and of the same size as in *P. argentipes*. There is a small spine between the spiracle and the 1st dorso-lateral hair.

In *P. papatasii*, vide Plate XXXVIII, fig 2, there are three hairy spines on the lateral surface of each abdominal, as well as, each thoracic segment—two being large and one small. The position of the smallest of the three hairs is different on the thoracic segments. In the abdominal segments the smallest hair is seen ventro-laterally—in the thoracic segments these small hairs are seen between the lateral hairs, closer to the dorso-lateral ones, and anterior to them, vide Plate XXXVIII, fig 6.

Ventrally—On the thoracic segments there are rows of hairy spines on the ventral surface. These spines are smaller than those on the dorsal surface and in all three species under consideration the spines situated between the central pair and the lateral one are much smaller than the others, and are also placed closer to the lateral spines.

The abdominal segments ventrally bear the pseudopods, seven in number, which have a pair of bristles, one on each side of the apex of that organ. On the penultimate segment which has no pseudopod there is a row of hairy spines. In '*P. minutus*' and *P. argentipes* there are four hairy spines and two simple spines—the spines being interposed between the central pair of hairs and their lateral fellows. In *P. papatasii* there are six hairy spines—two smaller than their fellows are placed

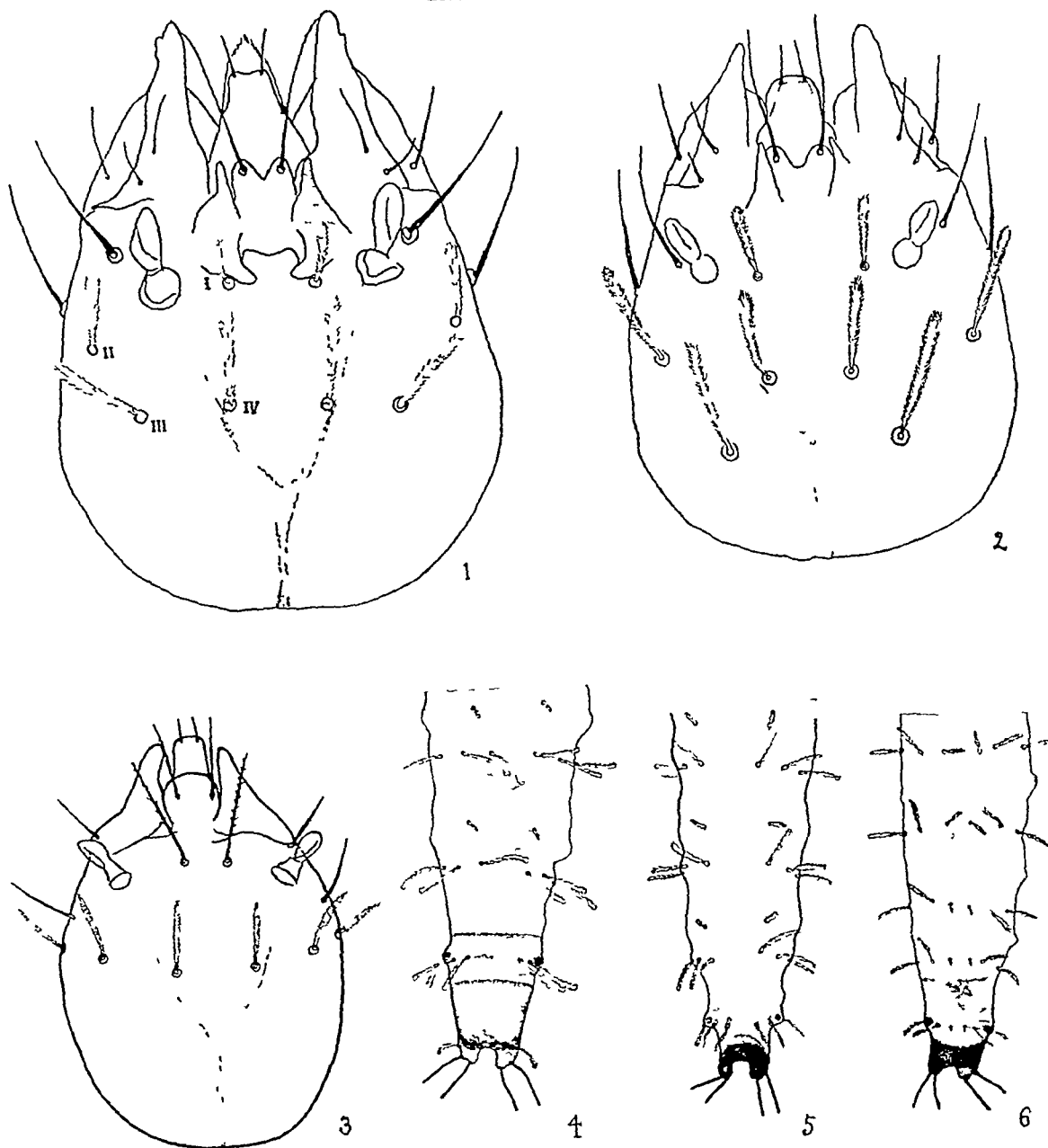


Fig 1 Dorsal view of head of larva of *P. papatasi*
 " 2 " " " " " " " *P. argentipes*
 " 3 " " " " " " " " *P. minutus*
 " 4 " " " " " " " " *P. papatasi*
 " 5 " " " " " " " " *P. argentipes*
 " 6 " " " " " " " " *P. minutus*

Showing trans
 verse rows of
 spines and
 short inter
 segmental
 spines

Explanation of Roman numbers, to Figs 1, 2 and 3 above

- No I Anterior hairy spines
 , II Antero lateral hairy spines
 , III Postero lateral hairy spines
 , IV Posterior hairy spines

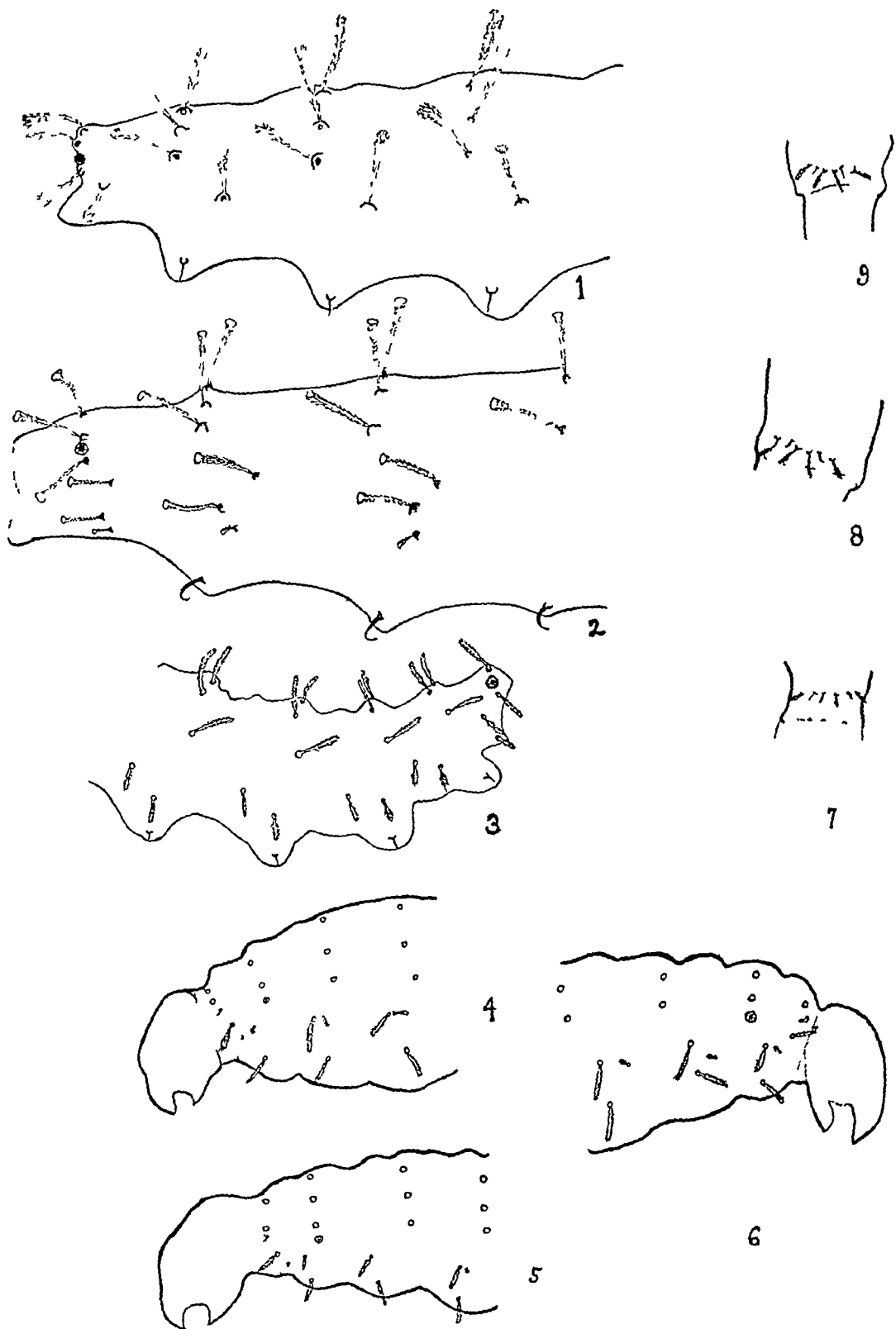


Fig 1	Lateral view of abdominal segments of larva of	<i>P. argentipes</i>	} Showing position of the spines
" 2	" " " " " " " "	<i>P. papatasu</i>	
" 3	" " " " " " " "	<i>P. minutus</i>	
" 4	" " " thoracic	<i>P. minutus</i>	} Showing position of the spines
" 5	" " " " " " " "	<i>P. argentipes</i>	
" 6	" " " " " " " "	<i>P. papatasu</i>	
" 7	Ventral " " penultimate	<i>P. minutus</i>	
" 8	" " " " " " " "	<i>P. papatasu</i>	
" 9	" " " " " " " "	<i>P. argentipes</i>	

slightly anterior to the others—in between the central pair and the lateral spines
Vide Plate XXXVIII, figs 7, 8 and 9

SUMMARY AND CONCLUSION

1 A method of differentiating the three species of *Phlebotomus* larvæ—*P. papatasi*, *P. argentipes* and '*P. minutus*'—met with in Bengal, is given

2 The differential characteristics are summarized in a tabular form in the Appendix

3 The position and character of the hairy spines in the different species of larvæ are shown in Plates XXXVII and XXXVIII

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APPENDIX

Table of differential characters of the three species of Phlebotomus larvæ

	THORACIC SEGMENTS			Head
	Dorsal surface	Lateral surface	Ventral surface	
		<i>P. papatasi</i>	<i>P. argentipes</i>	<i>P. minutus</i> (sensu lato)
		Postero lateral spines in line with posterior spines Anterior spines plumose	Postero lateral spines well behind posterior spines Anterior spines plumose	Postero lateral spines form with the posterior and antero-lateral spines an arc Anterior spines elongated and faintly branched and not plumose like the spine of the other species
		Row of six transversely placed hairy spines Three small hairy spines placed between the larger lateral spines. One small spine and one bristle placed ventral to dorso lateral spine	Row of six transversely placed hairy spines Two minute spines at base of dorso lateral spines of 2nd and 3rd segments. On 1st segment small spine between spiracle and dorso lateral spine and a minute spine ventral to the dorso lateral spine	Row of six transversely placed hairy spines, the two central spines being smaller than the others on the 1st segment Two small spines at bases of dorso lateral spines on 2nd and 3rd segments. A small spine between the spiracle and dorso lateral spine on 1st segment. A minute spine ventral to the dorso lateral spine
			Same as <i>P. papatasi</i>	Same as <i>P. papatasi</i>

ABDOMINAL SEGMENTS					
Dorsal surface	Rows of six spines placed transversely—seven rows of two small spines placed between transverse rows	Same as <i>P. pupatorum</i>	Row of six transverse spines on each segment. Central pair of spines get progressively smaller towards the last segment. * Small spines placed between the transverse rows very minute—not seen with low power		
Lateral surface	Three spines on each segment, one being much smaller than the other	One spine on each segment	Two spines on each segment		
Ventral surface	Pseudopods ⁴	Pseudopods	Pseudopods ⁴		
Dorsal surface	Well marked chitination of last segment, extends to lateral surface as well	Chitination on last segment not complete, has a convex border	Chitination of last segment involves two thirds of segment, and has a concave border		
Ventral surface	Six spines on penultimate segment—two placed anterior to the other four	Four spines and two bristles on penultimate segment—all in a line	Central pair of spines on penultimate segment very minute		

* This condition is also seen in 1st and 2nd instar larva of *P. argentipes*

STUDIES IN PERNICIOUS ANÆMIA OF PREGNANCY

Part VI

TROPICAL MACROCYTIC ANÆMIA AS A DEFICIENCY DISEASE, WITH SPECIAL REFERENCE TO THE VITAMIN B COMPLEX *

BY

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PERNICIOUS ANÆMIA has been defined as a disease due to the lack of the specific hormone, normally stored in the liver, necessary for the maturation of the megaloblasts of the bone-marrow. If this definition is accepted, tropical macrocytic anæmia will be included under this title, as well as Addison's or idiopathic pernicious anæmia, together with the macrocytic anæmias of sprue, idiopathic steatorrhœa and other similar conditions. Such a classification seems undesirable as it ignores symptoms other than the anæmia associated with these different conditions and obscures the variable ætiological factors so clearly defined by Castle and his colleagues (1929, 1930 and 1931). These workers formulated the conception of two factors, an intrinsic and an extrinsic, as necessary for the formation of the hæmopoietic principle present in liver, which permits of the orderly maturation of the megaloblast of the bone-marrow. By a series of brilliant experiments they have demonstrated that the intrinsic factor is present in normal human gastric juice and that the extrinsic factor is present in the protein of beef muscle and in the yeast extract 'marmite' or 'vegex' as it is called in America (Strauss and Castle, 1932). They further suggested that vitamin B₂ or some factor closely associated with it is the extrinsic factor (Strauss and Castle, 1932). In Addison's anæmia it is the intrinsic factor that is missing and a cure can only be effected if the extrinsic factor (beef muscle, marmite) is administered after incubation with the intrinsic factor in normal

* This work has been reported in brief in the *Lancet*, 1933, 1, p 1283. The expenses of the greater part of the work were paid by the Indian Research Fund Association.

gastric juice In the tropical disease, however, as far as can be judged by the presence of normal amounts of hydrochloric acid, the intrinsic factor is present in the gastric juice, but the extrinsic factor is missing from the diet It is impossible to be dogmatic about the presence of the intrinsic factor in the tropical cases on the existing evidence, its presence would be established if a case of undoubted Addison's anæmia were cured when treated with beef muscle that had been incubated with gastric juice from such a tropical case So far no case of undoubted Addison's pernicious anæmia has been available in Bombay The tropical form should be cured by giving the extrinsic factor alone, if that is the missing factor, and the rapid cures obtained when it is given in adequate doses in the form of marmite show that this is so (Wills, 1931) The difference from Addison's anæmia then seems to be such a fundamental one that it is proposed to continue at present to regard the conditions as distinct Obviously both Addison's anæmia and tropical macrocytic anæmia will respond to treatment with liver, administration of which, parenterally, is indicated in all very severe cases Liver contains the hæmopoietic principle, the end product of the interaction of the extrinsic and intrinsic factors, and when it is given parenterally, there can be no question of difficulty of digestion or of absorption

The tropical condition differs from Addison's pernicious anæmia in many respects, a full account of which appeared in a previous paper (Wills and Mehta, 1930) The most important differences are the earlier age incidence of tropical anæmia and its association with pregnancy, the presence of normal amounts of hydrochloric acid in the gastric contents, the absence of a raised indirect van den Bergh reaction and especially the absence of symptoms of nervous involvement These findings suggest a different ætiology, a supposition which is borne out by the result of treatment, and which will be discussed more fully below

It has been assumed that the extrinsic factor is missing in cases of tropical macrocytic anæmia, and it is interesting to review more fully the evidence in support of this assumption If the disease is a simple food deficiency, the world distribution of the condition should show its prevalence in areas where diets are known to be defective, and where other deficiency diseases are common China, the West Coast of Africa, and India, the areas where it is endemic, fulfil this condition and, moreover, the disease occurs among those sections of the community whose diet is known to be defective In India the disease is seen in two classes of people, those whose diet is limited by social or religious custom, and those whose diet is limited by poverty—the vast majority of the poorer town dwellers In a country largely vegetarian in habit, the nutritional value of a diet depends chiefly on the kind of cereal and amount of dairy produce eaten In the north wheat is the staple cereal and dairy produce is eaten in fair quantities, even in the towns, and here the disease is far less common than in the south where rice is the staple cereal and milk a luxury in many cities A dietetic survey (Wills and Talpade, 1930) in Bombay where the disease is common showed the diets of the hospital class of all communities to be inadequate in all respects except, in certain cases, in calories From the multiple deficiencies detected in the survey, it was impossible to indicate with any certainty, the nature of that one responsible for the condition of tropical macrocytic anæmia, but at the time it was thought that the relative deficiency of vitamins A and C might be the cause A trial with these vitamins, however, showed that they had no therapeutic value in the condition (Wills, 1931)

The key to the problem was found in animal experiments. A similar macrocytic anæmia was produced by dietetic means in monkeys (Wills and Bilimoria, 1932) and was cured by the administration of marmite. When this preparation was also tried in human cases it proved equally active (Wills, 1931). It was suggested that the curative agent might be either vitamin B₂ or some protein derivative.

The question whether this so-called 'pernicious anæmia of pregnancy' included here under the general term 'tropical macrocytic anæmia' is a clinical entity, distinct from the similar condition in men and non-pregnant women, must be considered. Strauss and Castle (1932*a*, 1932*b*) are of the opinion that anæmias in pregnancy have the same ætiology as those of the general non-pregnant population. In the author's opinion the same is true of the Indian cases. Severe anæmias of the microcytic, hypochromic type are as common in pregnancy as the macrocytic hyperchromic type, and both have the same ætiology and respond to the same therapeutic measures as the similar anæmias in the rest of the population. In the macrocytic cases the extra demands of pregnancy and lactation, as in other deficiency diseases, are precipitating causes. The amazing improvement frequently seen after delivery is paralleled in the immediate alleviation of symptoms *post partum* in many cases of osteomalacia, and is not therefore evidence that the anæmia arises directly out of the pregnant state. In both conditions symptoms may not appear till the strain of lactation begins. Gupta (1932), writing on pernicious anæmia of pregnancy, states that a similar anæmia is rare in men in Bengal. It is possible that the difference in diet between the sexes, common in all parts of India, is more marked in Bengal, or it is possible that the cases are missed. Certainly in Bombay the disease is rife among men. Among 113 consecutive cases of anæmia 44 or 39 per cent were males and, of the macrocytic cases of a total of 61, 32 or 52 per cent were males. These figures suggest that in spite of the predisposing effect of pregnancy, the incidence is practically the same in the two sexes. It seems probable, in fact, that a large proportion of the population of Bombay and other large cities in India lives on a diet relatively deficient in this anti-anæmia factor, and that it requires only a slight deterioration of the diet, such as occurs in hard times, or the added demands of pregnancy or some debilitating disease, to make the deficiency manifest.

The seasonal incidence of this anæmia remains to be explained. McSwiney (1927) in Calcutta and Balfour (1927) in Bombay have noted that the disease is commoner in the late autumn and winter. The diet of the poorer town dweller is so little affected by seasonal changes that it is hard to believe that minor variations in diet can be the explanation but at present the question must remain open till further work has been done.

The curative action of marmite in this tropical macrocytic anæmia was reported in a previous paper, and has been confirmed (Green-Armytage, 1932) and further extended to include other macrocytic anæmias including certain cases of Addison's pernicious anæmia (Goodall, 1932, Ungley, 1933, Davidson, 1932), idiopathic steatorrhœa (Vaughan and Hunter, 1932) and certain cases of sprue (Castle and Rhoads, 1932). Moreover, Strauss and Castle (1932) have shown that marmite contains the factor which they call extrinsic, and, when incubated with normal gastric juice, is curative in true relapse cases of idiopathic pernicious anæmia.

The condition of the blood and blood forming organs in pernicious anæmia is now universally recognized as a deficiency state, due to the lack of the hæmopoietic factor normally stored in the liver. There is, however, no evidence of a direct dietetic deficiency. The lack of the hæmopoietic principle is due to the absence of the intrinsic factor not to a lack of the extrinsic dietetic factor. All the evidence reported above points to the tropical disease being a food deficiency disease, the missing factor being Castle's extrinsic factor and therefore something present in both beef muscle protein and marmite. The work reported below was an attempt to determine further the nature of this extrinsic factor, with special reference to the vitamin B complex.

NATURE OF THE EXPERIMENT

The experiment planned was a clinical trial of the different fractions of the vitamin B complex with reference to their hæmopoietic action in cases of tropical macrocytic anæmia. The work was carried out in Bombay. The cases used were as far as possible uncomplicated, and included both men and women, certain of the latter being pregnant. The diagnosis was made on a full blood examination, including a Price-Jones curve. The methods used for the blood examinations were the same as in the previous work (Wills and Bilimoria, 1932). Only undoubted cases of macrocytic anæmia with high colour indices were used, this point is stressed as other workers in India include under the title of 'pernicious anæmia of pregnancy', besides the macrocytic cases, other severe anæmias with low colour indices, a confusion which leads to apparently contradictory results. In addition to the full blood examination, an alcohol test meal was given to practically all the cases, and the presence of free hydrochloric acid in normal amounts was demonstrated with only one exception, the findings are in agreement with earlier work (Wills and Mehta, 1930).

The patients were on the ordinary hospital diets, the severe ones on milk only. Except when on milk or light diet, when they received more milk than usual, the patients were receiving a diet very similar to their usual home one, rice and white bread forming the bulk of the food taken. Experience in other years had demonstrated in a tragic manner that these diets have no curative action. In an experience of more than 200 cases a natural remission except after delivery has never been seen, *cf* true Addison's pernicious anæmia, in which condition such remissions are quite frequent. Because of the severity of the cases (in 117 consecutive cases 45 per cent had a red cell count of under 1 million per c mm when first seen), and the absence of natural remissions, and because the patients will not stay more than a very short time in hospital, a control period was frequently impossible. However, a preparation was only considered active if it had been tried on patients who had either previously failed to respond to some other fraction, or else had been under observation and were known not to be healing. Conversely, a preparation was considered inactive only if the particular patients treated with it, without showing improvement, later responded to a preparation of known potency. Activity was judged by the magnitude of the reticulocyte response and the subsequent rate of blood regeneration. Cases that delivered during treatment were not used for test purposes.

PREPARATIONS TESTED

It is a pleasure to acknowledge the help given by Miss Harriette Chick, D.Sc., and her co-workers at the Lister Institute, London, both in the preparation and standardization of many of the extracts used. All the preparations that contained vitamin B₂ were standardized in terms of this vitamin, and given in doses that corresponded with the dose of marmite originally used. Later the doses of both marmite and the other extracts used were increased to ensure a maximal response in all cases.

Yeast powder—The Amritsar Distillery Co. very kindly gave a supply of dried yeast for clinical trial. The dose was 30 g. or more daily, given with milk and sugar in divided doses. The patients took it quite readily and in those doses it did not produce diarrhoea. The yeast was tested on rats for its vitamin B₂ potency by the method of Chick and Roscoe (1928) and found to be markedly less potent than an average sample of dried English brewer's yeast. This suggests that it might also have a lower content of other important biological factors.

Watery yeast extracts—These extracts were prepared at the Lister Institute from washed brewer's yeast, according to the method of Roscoe (1930). The watery extract is filtered off from the insoluble residue so that the final extract contains very much less protein than the original yeast. The daily dose was equivalent to 15, 30, or more grammes of dried yeast, i.e., the human dose was 75, 150, or more times the rat dose. The extract was used as originally prepared, and also after autoclaving for five hours at 120°C and 20 lbs. pressure.

Marmite—The same unflavoured marmite as used in the earlier work was placed at my disposal through the courtesy of the Marmite Food Extract Co. The use of this excluded the vegetables, added to the commercial extract for flavouring, as a possible source of the hæmopoietic factor. Dr. Chick had shown by tests on rats that this extract was comparable in vitamin B potency to dried yeast. The minimum human dose used was 40 times the rat's daily ration for the vitamin B complex. This extract was originally used in doses of 15 g. daily, but the dose was later increased to 30 g.

Insoluble and soluble fractions of marmite—A known weight of marmite was mixed with an approximately equal weight of water, brought to the boil and boiled for two minutes. On filtering this mixture an insoluble residue was left. This was given in daily doses equivalent to 35 g. of the original marmite to allow for any possible loss on the filter paper. The filtrate was given in doses equivalent to 30 g. of marmite.

Autoclaved marmite—A known weight of marmite was dissolved in water as described above, and then autoclaved for five hours at 120°C and 20 lbs. pressure. The original pH of this solution was 5.5. It was assumed that all the vitamin B₁ was destroyed by this treatment.

Alkaline autoclaved marmite—A watery solution was made as before, but was made alkaline to pH 9 before autoclaving. In an attempt to destroy the vitamin B₂ present, the mixture was heated for ten hours. The stability of vitamin B₂ has been found to vary according to the medium in which it is present, and it is possible that, although prolonged heating in an alkaline medium destroys this vitamin in watery yeast extracts (Chick and Copping, 1930), it may not be so destructive towards this vitamin in marmite (Guha, 1931). Roscoe (unpublished

work), however, has found vitamin B₂ in marmite to be only slightly more stable than in a watery yeast extract. It is therefore unlikely that more than traces remained in this preparation. Heating for ten hours brought down a heavy sticky precipitate that was very difficult to administer with the fluid fraction. For this reason the preparation was given in daily doses equivalent to 45 g of the original marmite to ensure an adequate intake.

Alcoholic extract of marmite—A weighed quantity of marmite was taken up with sufficient water to allow it to pour easily and then added to sufficient absolute alcohol to make the final concentration 80 per cent. This was done with varying quantities of water and alcohol as the nature of the precipitate varied with the total volume of fluid used. Dr. Malandkar of the Biochemical Department of the Haffkine Institute very kindly analysed the filtrates and showed that as long as the final concentration was 80 per cent, the amount of protein in the filtrate remained constant (Table I). The filtrate was originally given in daily doses equivalent to 30 g of marmite but later larger doses were used.

TABLE I

Nitrogen and protein in soluble fraction marmite and 80 per cent alcoholic filtrates

(Results calculated on 10 g marmite)

	Total nitrogen in grammes	Non protein nitrogen in grammes	Protein nitrogen in grammes	Total protein in grammes
Soluble fraction marmite	0.592	0.477	0.115	0.719
First alcoholic filtrate	0.307	0.250	0.057	0.356
Second alcoholic filtrate	0.294	0.237	0.057	0.356

Acid clay vitamins B₁ and B₄—Professor B. C. P. Jansen of Amsterdam kindly gave a supply of this preparation. It consists of vitamins B₁ and B₄ (Reader, 1930) from rice polishings adsorbed on to acid clay. The curative dose in beriberi is 2 g. to 4 g. daily, the larger dose was used in the present work.

Vitamin B₂ from egg white—This extract was prepared at the Lister Institute according to the method of Chick and co-workers (1930). Forty c.c. were equivalent to 1 g. dried yeast in vitamin B₂ potency, and so more than equivalent to the original dose of marmite. It was actually given in daily doses of 45 c.c. and 90 c.c.

CLINICAL TRIALS

The results are shown in Table II

Vitamins B₁ and B₄—Though all the evidence pointed to vitamin B₂ rather than vitamin B₁, being the active hæmopoietic factor in marmite, it was desirable to test the acid clay and thus exclude vitamins B₁ and B₄ if the material failed to cure. The acid clay was therefore given to two patients for a period of 10 days. Neither case responded, one remaining stationary and the other going downhill, but both reacted later to preparations of marmite (Chart 1). Further evidence that vitamin B₁ is not the active factor is afforded by the fact that cases treated with autoclaved marmite, in which the vitamin B₁ had been destroyed, responded immediately with a maximal reticulocyte crisis, followed by a typical remission. As vitamin B₄ is present in the acid clay as well as vitamin B₁, it also is excluded as the active factor in the cure of these anæmias.

Vitamin B₂.—In an earlier paper the author had suggested vitamin B₂ or some protein derivative as the curative factor in marmite, and Strauss and Castle (*loc cit*) also held that this vitamin or some substance closely related to it might be the extrinsic factor. In all the work reported up to date on this question, either beef protein or marmite ('vegex' in America) had been used, and it seemed very desirable to try some other source of vitamin B₂. The egg-white extract was therefore administered to two cases, one male and one female, in doses equivalent for vitamin B₂ to 16 and 32 g of marmite respectively. In neither case was there any response and the woman's condition deteriorated though both responded well later to autoclaved marmite (Chart 2). Additional evidence that vitamin B₂ is not the active hæmopoietic factor is afforded by the fact that watery yeast extracts of known vitamin B₂ potency were inactive and also yeast itself. Further, marmite, in which all but traces of vitamin B₂ had been destroyed by prolonged heating in an alkaline medium, still retained some curative power.

Watery yeast extracts—Ten typical cases of macrocytic anæmia were treated with watery yeast extracts of known potency for the B vitamins. All these cases failed to show any response. When these extracts were first used it had not been ascertained that vitamin B₂ was completely inactive curatively, it was rather assumed, owing to Castle's work, that extracts containing this vitamin would be as active as marmite. Two very severe cases were therefore treated with these extracts, as at the time liver extract was not procurable in the particular hospital where the patients were, unfortunately both went downhill rapidly and though liver extract was procured and given in large doses, both cases died. The other eight cases responded typically to active preparations of marmite (Chart 3).

Dried yeast powder—Five patients were treated with this preparation. As marmite is simply an autolysed yeast product it was anticipated that yeast, the parent material, would be active too. Dried yeast, however, in doses of 30 g or more daily had no hæmopoietic action. All the cases so treated failed to show any response though all responded well to active preparations later (Chart 4). This yeast was a poor source of vitamin B₂ and so possibly of other important biological factors. Later, however, two cases of typical macrocytic anæmia were treated in England with equivalent doses of a dried brewer's yeast of known good potency for the vitamin B complex, and also failed to improve, though both responded well to marmite later. Untreated dried yeast can therefore also be excluded as the source of the hæmopoietic factor.

TABLE II

Response to various preparations of yeast and the vitamin B complex

CASE NUMBER AND SEX																									
104 M	58 F	68 F	69 F	3 F	29 F	105 M	93 F	104 M	52 M	107 M	106 M														
FIRST PERIOD—DAILY DOSF OF SUBSTANCE ADMINISTERED																									
ACID CLAY	EGG WHITE	DRIED YEAST (INDIAN)	AUTOCLAVED WATERY YEAST EXTRACT	MARMITE		INSOLUBLE FRACTION OF MARMITE	80 PER CENT ALCOHOL FILTRATE.		AUTOCLAVED MARMITE	AUTOCLAVED ALKALINE MARMITE															
				15 g	30 g		Equivalent to 35 g marmite	Equivalent to 30 g marmite		30 g	Equivalent to 45 g marmite	Equivalent to 30 g marmite													
4 g	Equivalent to 32 g Luster yeast	30 g	Equivalent to 36 g Luster yeast	RBC	Ret	RBC	Ret	Equivalent to 30 g marmite	RBC	Ret	Equivalent to 30 g marmite														
* RBC	* Ret	RBC	Ret	RBC	Ret	RBC	Ret	RBC	Ret	RBC	Ret														
0	3 111	0.8	1 970	1.8	1 230	2.8	0.931	0.8	0.976	1.0	1 168	0.5	2 023	0.9	1 450	1.1	2 333	0.9	1 170	2.1	1 930	0.1	1 255	0.5	
2				0.5		1.8		2.3		0.9		0.7			2.0		0.6		1.5			2.6		0.3	
4				0.75	1 226	2.6		1.5		7.0		1.4			4.3	2 280	0.8		1 070	6.4		4.0		1.3	
6				1.5		1.0	0.883	2.7	1 210	26.0	15.6				2.5		1.0		16.0	2 040	9.5			3.0	
8				0.9	1 180	1.6		2.0		9.0	41.0	20.0	0.9		0.7		4.5		1 490	35.3		2.6		1 320	4.0
10	2 333	0.8	1 030	0.7		3.1	0.902	2.4	1 500†		1 990	25.0	0.5	1 797	20.0	2 100	3.8		8.0	2 315	1.9			5.1	

Days of treatment

SECOND PERIOD—DAILY DOSE OF SUBSTANCE ADMINISTERED

Days of treatment	MARMITE		AUTOCLAVED MARMITE		MARMITE		AUTOCLAVED MARMITE		AS ABOVE		SOLUTION FRACTION MARMITE		MARMITE		AS ABOVE		AS ABOVE	
	30 g		30 g		30 g		30 g		30 g		Equivalent to 30 g marmite		30 g		30 g		30 g	
	RBC	Ret	RBC	Ret	RBC	Ret	RBC	Ret	RBC	Ret	RBC	Ret	RBC	Ret	RBC	Ret	RBC	Ret
0	2500	08†	1810	07	1180	13	0090	21			2070	05	2590	08				52
2		07		10		35		21				07		07				16
4	2809	91		25		40		10				11	2808	91				60
6		110	1560	120		130	1015	120	2030	270	2780	110		110				66
8		110		160	1250	320		280			120			110			1187	94
10	3610	170	2290	30		100		156			3250	30	3610	170	2010			
20	3840		2860		2370		2850		3556					3840	1100			
40	4670				1525		1297		4010 § (30 days)				4670				2680	

Note.—* RBC = Red blood cells in millions per c mm Ret = Percentage of reticulocytes in total red cells

† Delivered 5 days later

‡ Treated with alcohol extract for 12 days between first and second period

|| Gave maximal response to marmite later

§ Delivered 7 days later

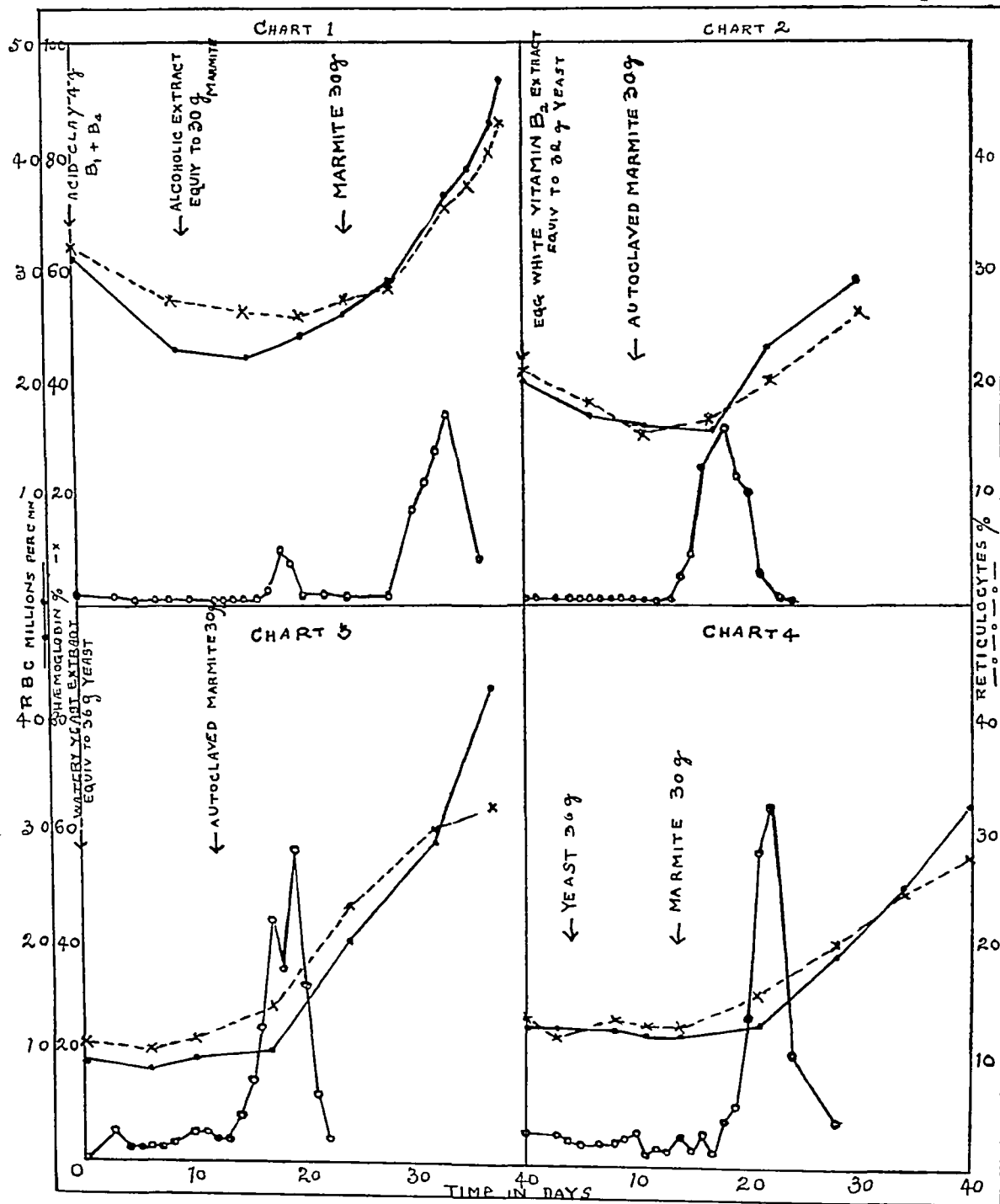
Marmite—Ten cases were treated with unaltered marmite to confirm the previous work and also to show that cases that had failed to respond to other preparations would respond to treatment with marmite. There were two failures in this series that must be considered in view of certain criticism of this method of treatment. The one case was of special interest as she was the first ever treated with marmite (1931). She then made a maximal response and was discharged cured, only to relapse at home. She was a poor Brahmin widow and therefore a strict vegetarian. She was readmitted and again discharged cured, but once more relapsed and was again admitted, critically ill with a red cell count of about a million. Treated with marmite she made a submaximal response and seemed to be improving when she developed a septic mouth and acute parotitis, went downhill rapidly and died three days later, in spite of intravenous liver treatment. Sepsis is known to inhibit the hæmopoietic effect of both liver and marmite, but the poor initial response in the third attack compared with the maximal responses on the two previous occasions suggests that the marrow may have been becoming aplastic. The second case was associated with intractable diarrhoea of some years standing, there was no response to either marmite or liver extract by mouth, neither preparation being tolerated in any but small doses. The other eight cases on marmite made maximal responses. One, a pregnant woman, remained in hospital long enough for her red cell count to rise from 1 to 4 millions per c mm when she was delivered of a healthy full term infant and seven days later her red cell count was 4.6 millions per c mm (Chart 5).

Before leaving the question of the activity of marmite in these cases of tropical macrocytic anæmia, it is interesting to note the results in 10 cases treated at the same time with liver extracts. Here again the cases were on the whole very severe and there were five deaths, or these five fatal cases, two were pregnant women with severe urinary sepsis, 2 were men both critically ill when admitted with red cell counts below 0.7 million per c mm who failed to respond to vigorous liver treatment by both the intravenous and intramuscular routes, and the fifth case has been reported above. It is thus obvious that both liver and marmite therapy will continue to give some negative results as long as the patients fail to report in the earlier stages of the disease, but as a whole the results with both liver and marmite in tropical macrocytic anæmia compare very favourably with those obtained with liver therapy in Addison's pernicious anæmia.

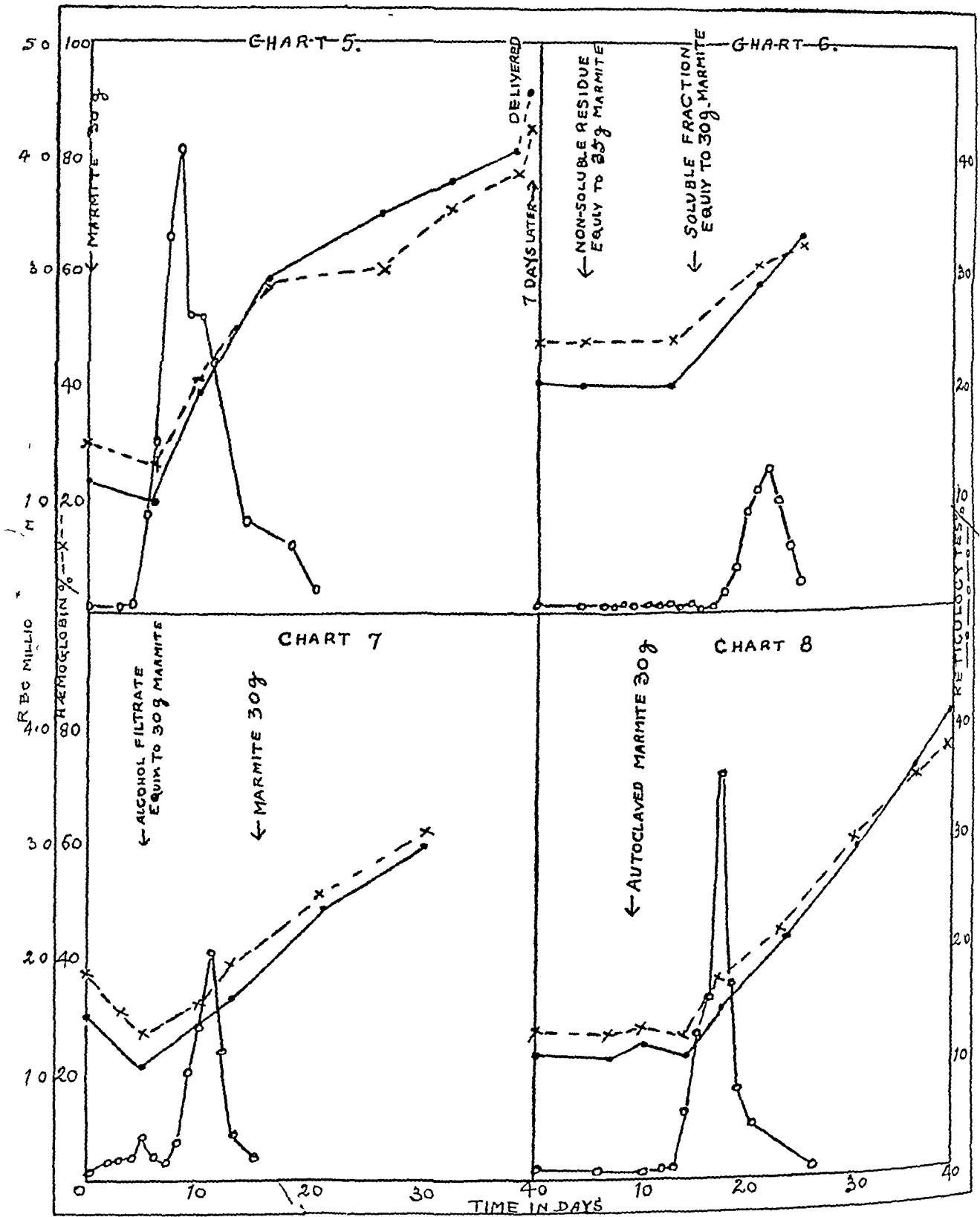
After confirming the previous work with marmite an attempt was made to determine the nature of its hæmopoietic fraction. The work reported above had excluded the vitamin B complex. The trial of soluble and insoluble fractions showed the active principle to be water soluble (Chart 6). It was also soluble in 80 per cent alcohol (Chart 7) though some of the potency of the original material was lost as all the cases did not respond to doses equivalent to 30 g. of marmite. The loss of potency may depend on the physical nature of the precipitate and the amount of washing.

The active fraction survived autoclaving for five hours at 120°C and 20 lbs pressure, at an initial pH of 5.5. Five cases treated with marmite so heated healed so dramatically that an impression was derived that the potency of the preparation had actually been increased by the treatment (Chart 8), certainly there had been no loss of hæmopoietic factor. Two further cases treated with this preparation did not respond, one, a male admitted in a critical state with a red cell count of

CHARTS 1 to 4



CHARTS 5 to 8.



580,000 per c mm, died on the second day of treatment and the other, a middle-aged man admitted with a red cell count of 883,000 per c mm and an abscess in the external auditory meatus, showed no improvement for 10 days. At the end of that time, when the abscess had drained, he received liver extract and made a maximal response.

There was a considerable loss of potency after autoclaving marmite at an initial pH of 9.0 for 10 hours at 120°C and 20 lbs pressure. Such treatment brought down a heavy, sticky precipitate, very difficult to administer and also extremely unpleasant to the taste. In doses equivalent to 30 g marmite there was no response and with larger doses, equivalent to 45 g, the results were variable, in one case so treated there was a good response, and in another only a submaximal one, though this case gave a maximal response to marmite later.

DISCUSSION

The work reported above was planned to confirm the previous findings on the therapeutic value of marmite in tropical macrocytic anaemia, and to attempt to determine the nature of the dietetic deficiency believed to give rise to the condition.

The therapeutic value of marmite has been amply confirmed by the author and by other workers. The negative results of Mudaliar and Rao (1932) are difficult to understand if the cases treated by them were of the same type as those described by the author. It should, however, be noted that the seasonal incidence is totally different from that observed in Bombay and Calcutta, that they report a large percentage of cases with either hypochlorhydria or achlorhydria whereas the author's cases had as a whole a normal gastric acidity, and finally that they include under the title of 'pernicious anaemia of pregnancy' cases with low colour indices, which, in the author's experience, would not respond to either liver or marmite treatment. Till a full record of the cases is published, it is difficult to assess the significance of Mudaliar and Rao's findings.

As regards the exact nature of the dietetic deficiency the vitamin B complex has been excluded as the active fraction in marmite and as Castle's extrinsic factor. Dried yeast, watery yeast extracts, and preparations of the B vitamins from other sources (egg white and rice polishings) have been shown to be completely inactive. It has also been shown that egg white incubated with normal gastric juice was completely inactive in a case of true Addison's anaemia when given in doses corresponding in vitamin B₂ potency with the curative dose of marmite (Wills, 1933). This work has been confirmed by Groen working in Professor Snapper's laboratory in Amsterdam (personal communication to be published shortly). This worker used marmite, yeast, and egg white as Castle's extrinsic factor and gave them after incubation with normal gastric juice, to cases of idiopathic pernicious anaemia. He too found that marmite was active, whereas yeast and egg white were inactive when given in this manner.

The hæmopoietic factor in marmite has been found to have the following characteristics. It is water soluble, heat stable in acid medium and partially resistant to autoclaving in an alkaline medium, it is not precipitated or inactivated by 80 per cent alcohol. Marmite is an autolysed yeast extract, and it is possible that in the process of autodigestion the active principle is formed, but it seems unlikely

that it is the same as the hæmopoietic factor in liver. The majority of cases of true Addison's anæmia do not respond to untreated marmite, and it seems more likely that those cases of Addison's anæmias that do so still have some intrinsic factor present in their gastric juice. It is difficult to understand the inactivity of yeast but before it can be ascertained with certainty that the active fraction is really absent, only appearing after autolysis, cases must be treated with crushed yeast cells, since the intact cell of ordinary brewer's yeast is only digested with difficulty by normal gastric juice, even after prolonged incubation. Watery yeast extracts contain so little of the solid yeast that the active fraction may well be discarded with the insoluble residue. It is obvious that much more work is necessary before the nature of this hæmopoietic factor or its distribution in nature is known.

There seems, however, sufficient evidence to warrant the assumption that the disease, both in pregnant and non-pregnant women and men, is a simple food deficiency and not a conditioned food deficiency as in true pernicious anæmia. The evidence for this has already been discussed but it is important to stress the point particularly from the point of view of preventive medicine. As a prophylactic measure, in areas where the condition is endemic, education and public health policy should encourage the consumption of protein of good biological value, especially meat, and, further, for a vegetarian population, the use of some autolysed yeast product such as marmite. Till the exact nature of the extrinsic hæmopoietic factor is determined and its distribution in food-stuffs known, the advice cannot be more exact, but a diet including adequate quantities of either beef muscle protein or marmite should prevent the onset of symptoms, except in those rare cases where the intrinsic factor is missing.

SUMMARY

(1) Evidence is adduced and discussed that tropical macrocytic anæmia is a simple condition of dietary deficiency.

(2) The curative action of marmite in this condition already reported has been confirmed.

(3) The nature of the hæmopoietic factor was investigated by the use of various specially prepared fractions of the vitamin B complex.

(4) By this means the recognized constituents of the vitamin B complex were excluded.

(5) The hæmopoietic fraction of marmite was found to be water soluble and heat stable in acid medium and not to be precipitated or inactivated by 80 per cent alcohol.

ACKNOWLEDGMENTS

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AGES OF EPIPHYSIAL UNION AT THE ELBOW AND WRIST JOINTS AMONGST INDIANS

BY

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AND

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[Received for publication, October 16, 1933]

THIS investigation was carried out in the Radiology Section of King George's Medical College, Lucknow, the expenses of the Inquiry being defrayed from a grant from the Captain Kunwar Inderjit Singh Research Scholarship Fund. It was undertaken to ascertain whether the ages of union of epiphyses amongst Indians differ from those of Europeans (as given in the different textbooks of anatomy), on account of the commonly held view that the Indians reach maturity at an earlier period of life, and incidentally to supply the medical jurist, who has to depend at present on European textbooks, with figures more especially applicable to Indians in the many medico-legal cases where the question of age frequently crops up. The radiological method of studying the unions was preferred to the purely anatomical, and observations could be made on a much larger number of subjects of known age, who were otherwise normal, and the figures thus arrived at would naturally give more correct averages than a study in the anatomical laboratory findings.

We with Gaoth unfortunately carry out these observations over a large enough number that, with to enable us to give fully authoritative figures, as on account of prejudicial age at parents and for other reasons we could with great difficulty win over the nation of only one local school (Hussainabad High School). The results, throw interesting light on the subject and give figures comparable to those of workers in other countries and on other races. In all 52 boys of ages from 15 to 20 years were examined. The total number of skiagrams taken was observed. These were in many cases taken in both antero-posterior and lateral views to make certain about the union.

The point at which union may be considered to be complete is rather difficult to define in a skiagram. For the purposes of our investigation we counted an epiphysis as united only if the epiphysial space was, in bony architecture and density, indistinguishable from the epiphysis or diaphysis in its neighbourhood. Cases of recent union where a line was still seen in the place of epiphysial cartilage were not classified as united.

Our observations are of course confined only to males and, on account of the generally accepted view that females show union earlier (Pryor, 1928) by two years or more, it is probable that union in the case of Indian girls must be taking place at ages that would be surprisingly young compared to the teaching of the standard anatomical textbooks.

We have divided the students in four age groups of 16-17, 17-18, 18-19 and 19-20. The number of boys examined in each group is respectively 15, 21, 11 and 1. The group of 19-20 is too small for averages, and accordingly the average per cent has been confined to the age groups 16-17, 17-18 and 18-19.

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discussion in this pa
18-19 years

The results of this
and III

We shall now attempt to compare our results
observers for other races

quiry are given in the different age groups in Tables I, II

compare our results for Indians with those of recent

US

LOWER END OF THE HUMERUS

According to Paterson (1929) this joins the diaphysis at 19 years. Parsons (1927) state, 'their junctions to the shaft was first noticed at 16 years 4 months and from thence onwards in our observations' In Derry (1931) state that the distal end of the humerus is completely joined in 92.6 per cent of cases by the time the subject has reached 17 years of age. They further state that unlike the finding of Davies and Parsons, 35 per cent of boys have no figures between 14 and 15 examined by them show this union. We have no figures for age groups earlier than 16, but according to our observations 87 per cent show union by the time the boy has reached 17. Our results are therefore much more in agreement with Sidhom and Derry than English observers, obtained by more especially Paterson. They are much nearer the result of Borovansky and Hnevkovsky (1929) for Prague boys than those of Paterson or Paterson for English boys.

ysis at 19. Davies and Parsons noticed at 16 years 4 months. Egyptians, Sidhom and Derry completely joined in 92.6 years of age. They have no figures per cent of boys have no figures observations 87 per cent are therefore English observers, obtained by other Davies

MEDIAL EPICONDYLE

All authors agree that this unites later than the rest of the shaft. Our observations also support this view. There are again differences between English, Prague and Egyptian boys. Paterson writes, 'join the shaft somewhere between 18-21'. Davies and Parsons, on the other hand, state, 'it remains separate up to the 20th year'. It is an open question whether the epicondyle joins the shaft before or after the head of the humerus. Our statement is altogether and positively against our observations.

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Hnevkovsky write, 'fusion is accomplished in 67 per cent of all cases at 17 and in 95 per cent of all cases at 19 years of age' Egyptian figures are somewhat similar. Prague figures closely agree with our observations. The medial epicondyle is united in 67 per cent of cases in the 16-17 group and 90 per cent of cases in the 17-18 group amongst Indians.

UPPER END OF RADIUS

According to Paterson this joins the shaft very constantly during the 18th or 19th year, i.e., during the age group 18-19 or 19-20. Paterson, Davies and Parsons put the average age of junction as 15th to the 16th year. The Continental observers above quoted state, 'fusion with the diaphyses begins after 14 years of age and is accomplished in the 18th year'. Our figures are in agreement with those of Sidhom and Derry for Egyptians. There is no doubt that the fusion must start much earlier than 16 years, although the maximum increase is in the 17-18 group, as even in the 16-17 group ten out of fifteen show this union.

UPPER END ULNA

Paterson again puts this union very late—at about 19 years. Davies and Parsons consider that this union takes place a little later than that suggested by textbooks and would place it in the 17th year. Our figures show that it takes place a little earlier than that of the upper end of the radius in Indians and seem to agree here with the observations of Davies and Parsons.

LOWER END OF THE RADIUS AND THE ULNA

We are discussing these two together as our figures, unlike others for both of these bones are much the same and are very different from those given by any of the other authors quoted above. Paterson gives the age of union as 21, Davies and Parsons put it at 20. Sidhom and Derry state, 'only 8 per cent were united in the 16-17 group', while our figures (given in the summary) give the percentage of union in that age group as 40. Whereas only 50 per cent of the Egyptian cases showed union in the 18-19 group, as many as 73 per cent of our cases were united in the corresponding period—a figure reached by the Egyptians in the next, i.e., 19-20 group. Hence our observations point to the age of union amongst Indians being a little lower than in Egyptians—at about 19 years of age. This is in close agreement with the findings of Flecker (1932) for Australians. This author comparing his results with Galstaun's (1930) states, 'Thus it would appear in Galstaun's Hindu series, that, with the exception of a few instances, fusion does not take place generally at an earlier age than in this Australian series of European descent'.

SUMMARY

Our observations can be tabulated as follows in the form of percentage union in different age groups. The figures within brackets are similar percentages.

TABLE II—(17-18)

Number	RADIUS		ULNA		HUMERUS	
	Upper	Lower	Upper	Lower	Medial epicondyle	Lower
1	+	+	+	+	+	+
2	+	—	+	—	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	—	+	—	+	+
6	+	+	+	+	+	+
7	+	—	+	—	+	+
8	—	—	+	—	—	+
9	—	—	+	+	+	+
10	+	+	+	+	+	+
11	+	—	+	—	+	+
12	+	+	+	+	+	+
13	+	—	+	—	+	+
14	—	—	—	—	—	—
15	+	—	+	—	+	—
16	+	+	+	+	+	+
17	+	+	+	+	+	+
18	+	+	+	+	+	+
19	+	+	+	+	+	+
20	—	—	+	—	+	—
21	+	+	+	+	+	+

TABLE III—(18 19)

Number	RADIUS		ULNA		HUMERUS	
	Upper	Lower	Upper	Lower	Medial epicondyle	Lower
1	+	+	+	+	+	+
2	—	—	+	—	+	+
3	+	+	+	+	+	+
4	+	—	+	—	—	+
5	+	+	+	+	+	+
6	+	+	+	—	+	+
7	+	+	+	+	+	+
8	+	—	+	—	+	+
9	+	+	+	+	+	+
10	+	+	+	+	+	+
11	+	+	+	+	+	+

ACTION OF *B COLI* ON CONJUGATED BILE ACIDS

BY

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[Received for publication, November 24, 1933]

IN order to obtain free bile acids from the state of conjugation with amino-acids in which they occur in the bile, one has to depend exclusively on the method of hydrolysing with hot and strong alkali. The method is rather drastic and besides, the possibility of some isomeric changes in the bile acid molecule during the hydrolysis is not excluded. The question, if and how far it is possible to bring about a cleavage of the conjugated bile acids with enzymes and bacteria, especially those of the intestinal tract, is therefore very important from the stand-point of preparative as well as biological chemistry.

Smorodinzew (1922) tried the action of the enzyme histozym on conjugated bile acids and claimed that he obtained a cleavage. But the extremely crude methods employed by Smorodinzew make any qualitative, not to speak of a quantitative, estimation of the results of his experiments impossible, for it is not possible to distinguish taurocholic and glycocholic acids from cholic acid either on the ground of the solubility of the barium-salt or with the iodine reaction of Mylius, as has been done by Smorodinzew.

Grassmann and Basu (1931) tried the action of pancreatic trypsin, of glycerine extracts of pancreas, mucous membrane of the intestine, fresh liver, and kidney, of papain and of an aqueous extract of *Aspergillus oryzae* on conjugated bile acids. In all cases a negative result was obtained except in the case of extracts of kidney and *Aspergillus oryzae* in which case a slight cleavage was obtained.

As it is generally supposed that the conjugated bile salts are hydrolysed during their passage through the intestine, it was thought advisable to study the action of *B coli*, an intestinal bacteria capable of bringing about various kinds of reactions, on conjugated bile acids.

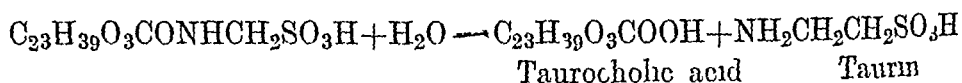
EXPERIMENTAL

A pure culture of *B. coli* on agar was obtained from Messrs Bengal Immunity Co., Calcutta. This *B. coli* was sub-cultured thrice successively in nutrient broth agar. The nutrient broth was prepared by dissolving 5 g of meat extract, 2 g of peptone (Witte), 0.2 g sodium chloride and making up to 1,000 c.c. with water. This was sterilized in the autoclave for 15 minutes under 1 atmosphere pressure. Two g of sodium chloride were then added and the solution filtered. Five g of agar were dissolved in 100 c.c. of this solution by heating in steam, and 5 c.c. of this nutrient broth agar were taken in each test-tube which was sterilized in an autoclave before a sub-culture of *B. coli* was made in it.

In order to make the *B. coli* adaptive to bile salt it was sub-cultured successively thrice in a medium containing 20 g peptone, 5 g sodium taurocholate and 10 g lactose per litre. In order to avoid the decomposition of the bile salt this medium was sterilized in steam on three successive days for seven hours each day.

Next to get the *B. coli* accustomed to a bile salt medium without lactose it was again sub-cultured thrice successively in a medium containing 20 g peptone and 5 g sodium taurocholate per litre. At first the growth of *B. coli* was poor but on the third occasion the growth was very vigorous. Five hundred c.c. of this sterilized bile salt medium without lactose were then inoculated with this *B. coli* and placed in the incubator at 37°C for a week when a very good growth of *B. coli* was obtained. The solution containing the bacteria was then centrifuged at a speed of 5,000 revolutions per minute. The *B. coli* collected at the bottom of the tube. It was then repeatedly washed with physiological sodium chloride solution by centrifuging and ultimately shaken up to 100 c.c. with physiological sodium chloride solution. From this a definite volume, 5 c.c., was pipetted off for each experiment.

The conjugated bile salt on which the action of *B. coli* was tried was sodium taurocholate. In case of a cleavage by bacterial action, the following reaction should take place —



With the progress of cleavage the acidity should therefore increase and should be capable of measurement by Willstatter's method of estimating amino-acids by titrating with alcoholic caustic potash in 90 per cent alcoholic solution, using an indicator like thymolphthalein whose end-point lies far off in the alkaline region (pH 10–11).

The experimental procedure was therefore as follows. A stock solution of 15 g sodium taurocholate in 250 c.c. was prepared. As reaction vessels, measuring flasks of 25 c.c. capacity were used, into each of which 15 c.c. of the taurocholate solution at 37°C were taken. This was then brought to the desired pH by addition of the requisite number of drops of acid or alkali using Clarke's and Lubbock's series of indicators. Two c.c. of M/10 phosphate buffer at 37°C were then added and next 5 c.c. of the *B. coli* suspension also at 37°C. The volume was made up to 25 c.c., and the flasks maintained at 37°C in a thermostat and shaken from time to time. Five c.c. of the solution were pipetted off from time to time, 50 c.c. of alcohol added and titrated from a micro-burette with N/20 alcoholic potash using thymolphthalein as the indicator till a permanent blue colour was obtained.

RESULTS

The following series indicate the typical results —

TABLE I

Control
Sodium taurocholate solution
alone

Time in hours	c c N/20 KOH
0	3.69
24	3.69
72	3.69
96	3.69

TABLE II

Sodium taurocholate and
B. coli suspension
without buffer

Time in hours	c c N/20 KOH
0	3.66
24	3.54
72	3.44
96	3.40

TABLE III

Sodium taurocholate at pH 6.81,
phosphate buffer pH 6.8 and
B. coli suspension

Time in hours	c c N/20 KOH
0	4.25
24	4.25
72	4.25
96	4.25

TABLE IV

Sodium taurocholate at pH 8.03,
phosphate buffer pH 8.03 and
B. coli suspension

Time in hours	c c N/20 KOH
0	3.94
24	3.56
72	3.46
96	3.16

DISCUSSION

It would be apparent from the above tables that *B. coli* brings about no cleavage of the conjugated bile acids. On the other hand Tables IV and II indicate that in alkaline medium (sodium taurocholate solution itself is very slightly alkaline) the bacteria decompose a slight portion of the conjugated bile acids, as a result of which the titre gradually falls.

Schiff (1870) first postulated the theory of a bile cycle according to which the bile is resorbed from intestine and finds its way back to the gall-bladder through portal vein and liver. It is generally held [e.g., Halliburton (1921)], that in its passage through the intestine the conjugated bile acid is broken up into the constituent bile and amino-acids and is formed again in the liver. The previous

investigations of Grassmann and Basu (*loc cit.*) already indicated, and also the present work makes it abundantly clear, that the bile cycle involves a cycle of the conjugated bile salts, and that the latter are not hydrolysed during their passage through intestine though a small fraction might be bacterially decomposed

Our grateful thanks are due to Professor J C Ghosh for his keen interest in the work.

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SPLENIC ENLARGEMENT IN SOUTH INDIA A STUDY BASED ON POST-MORTEM RECORDS

BY

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No figures exist of the causes of splenic enlargement for South India and this study has therefore been undertaken in order to get an idea of the factors that contribute to its incidence here. This is based on the post-mortem records of the Government General Hospital, Madras, and the Government Royapuram Hospital, Madras. The former figures date from the year 1889 although a Pathological Department was first created in the Madras Medical College earlier. The figures range for a period of 40 years from 1889 to 1929 for the General Hospital and to 1933 for the Royapuram Hospital. Most of these figures relate to the records of pathologists who held office as Professor of Pathology in the Medical College. The records of more recent years refer to work with which the writer was more intimately connected both at the Medical College and the Royapuram Medical School. In many instances these post-mortem findings are amplified by microscopic examination of smears and histological sections even dating back to the nineties. One is, at the outset, compelled to pay a tribute to the work of these early pathologists for the completeness of their observations and records.

The total number of autopsies analysed is 5,034. The normal weight of the spleen is taken to be between 3 oz and 7 oz following the figures of this presidency for adults for medico-legal autopsies. Anything above 7 oz is taken as enlargement. This therefore corresponds to the 200 g taken by De (1932) as the minimum for enlargement in Bengal. Of these 5,034 autopsies the total number of cases showing enlargement of the spleen was only 1,101 or 21.9 per cent, a figure which compares quite favourably with 54 per cent in De's figures for Bengal and 40 per cent in Gharpure's figures (1933) for Bombay. De's figures and these I have taken are on the same standard, but Gharpure takes 150 g as the minimum for enlargement, and so his figures for Bombay, judged by our standards, are a little too high. These enlarged spleens are further classified into four groups closely following De's standards.

Thus, 200 g to 500 g slight enlargement,
500 g to 1,000 g moderate enlargement,
1,000 g to 1,500 g marked enlargement,
over 1,500 g extreme enlargement

The analysis that follows is based upon well recognized pathological types and, unlike De's analysis, is not based on clinical history or clinical diagnosis.

Group I includes those cases of acute enlargement with softening that go under the name of 'septic spleen' or the acute splenic tumour of the clinician. I have included a sub-group under the term 'acute congestion' which I believe to be slightly different. Under another sub-group is the solitary instance of 'abscess of the spleen'.

Group II includes cases of 'chronic venous congestion' mostly due to cardiac failure of the congestive type from myocardial, valvular, or pericardial disease. There is one instance of congestion and moderate enlargement due to thrombosis of the portal vein.

Group III of splenic enlargement associated with cirrhotic changes in the liver. I have put down under a separate head, since the process appears to be slightly different from simple venous congestion.

Group IV includes diseases of hæmatopoietic system, such as leukaemia.

Group V includes the chronic hyperplasias such as those due to the infective granulomata.

Group VI is a miscellaneous group where rare causes of splenic enlargement are included.

In Group VII I have put down all unclassified cases.

GROUP I A

Septic spleens Total cases 399

Lobar pneumonia	71
Bronchopneumonia including abscess and gangrene of the lung	44
Enteric fever	15
Septicæmia and pyæmia	48
Acute peritonitis	23
Pleurisy and empyema	12
Acute pericarditis	3
Acute endocarditis	9
Acute meningitis	7
Dysentery, unclassified	6
Dysentery, bacillary	9
Dysentery, amœbic	18
Abscess of the liver	23
Pyelonephritis	8
Burns	5
Influenza and influenzal pneumonia	6
Miscellaneous infective conditions	90
Acute yellow atrophy	2

This first group consists of 399 instances of acute septic spleen. The enlargement is mostly slight (200 g. to 500 g.), and there are a few cases where it is moderate (500 g. to 1,000 g.). The appearance of the spleen in these cases was one of hyperplastic softening met with typically in acute infectious fevers, especially in septicæmias. Evans' morphological division into 'red septic spleen' characteristic of simple hyperplasia and met with in typhoid fever, and 'grey septic spleen' of leucocytic emigration of septicæmia, is not borne out by these records. The distinction that is possible here is to divide these septic spleens into a dark red type,

where there is more of hyperplasia and congestion, and a pale red type where there is little hyperplasia but leucocytic collections are more in evidence. On analysis of these 399 cases the commonest cause of this type of splenic enlargement was seen to be lobar pneumonia. This is probably due to the large number of such cases that come to autopsy. Of enteric fever, which bulks largely in the eyes of the clinician, we have only 15 cases. This is probably due to the difficulty in obtaining autopsies in this type. Unusual figures in this group are the number of cases of abscess of the liver. Of the 23 cases, only three were abscesses due to pyogenic infection, the rest were all amœbic abscesses. Of the dysenteries, amœbic dysentery has a high figure. Malaria as a cause of enlargement in these conditions is excluded by the appearance of the spleen. Dysentery complicating such conditions as malaria and kala-azar is classified under the latter heads. I have not been able to discover malarial pigment or parasites in those cases I have personally examined. Eight cases of pyelonephritis are of interest to the surgeon. They raise the possibility of hæmatogenous infection of the kidney. Pleurisy with effusion and empyema causing splenic enlargement is another feature that is worthy of note. The enlargement was distinct but slight, except in one case where the spleen weighed over 500 g. There were six cases of influenza and influenzal bronchopneumonia where a slight degree of enlargement was met with, probably owing to the severity of the infective process. Splenic enlargement in influenza is seldom met with by the clinician except in the gastro-intestinal types, but acute congestion of the spleen has often been reported in autopsies.

GROUP I B

Acute congestion of the spleen Total cases 30

Shock after severe fracture and injuries	22
Poisoning	6
Sunstroke	1
Heat-stroke	1

Under the heading of acute congestion, I have included a sub-group, where the nature of the splenic enlargement is slightly different from septic hyperplasia. This type I believe to be due to sudden vascular engorgement from alterations in the splanchnic circulation. Many would include this group under the heading of septic spleens, but the ætiological factors appear to be different. In a few of these types I have examined, endothelial hyperplasia was not prominent, but areas of congestion and hæmorrhage into the pulp were met with. Out of thirty cases of this sub-group, primary shock following severe fractures, such as fractures of the spine, skull, etc., and very severe injuries, were responsible for 22 cases, and six were due to poisoning of various types, such as acute alcoholic intoxication, where the cause of the enlargement is more obscure. Malarial and other types of chronic hyperplasia that might have been coincident are not included in this group.

GROUP I C

Abscess of the spleen Total case 1

Under this sub-group I have an instance of abscess of the spleen where coincident dysenteric lesions would lead one to suspect an amœbic abscess of the spleen. There is, however, no further evidence on this point.

GROUP II A

Chronic venous congestion of the spleen with enlargement Total cases 63.

Slight enlargement (200 g to 500 g)	. 53
Moderate enlargement (500 g to 1,000 g)	. 7
Weight not recorded	3

Only slight enlargement was met with in most cases of chronic venous congestion. The spleen in these cases was hard, elastic, almost purple in colour, with a firm pulp, well-marked trabeculae, and a thickened capsule which was often slate grey in colour. Histologically, the picture was one of increased prominence of the sinuses, which became distended with blood. There was increased fibrosis of the trabeculae and increase in the elastic tissue. Most of these cases were due to cardiac failure. One case in this group is worthy of interest. It occurred in beri-beri where the right-sided cardiac failure had caused an enlargement of over 600 g.

GROUP II B

Chronic venous congestion due to thrombosis of the splenic vein Total case 1

GROUP II C

Chronic venous congestion due to thrombosis of the portal vein Total case 1

Chronic venous congestion from thrombosis of the splenic vein caused moderate enlargement in one case and thrombosis of the portal vein caused marked enlargement in another.

GROUP III

Splenic enlargement associated with cirrhosis of the liver

Total cases 101 (+2 syndrome of Banti)

Portal cirrhosis of the liver	92
Slight enlargement	55
Moderate enlargement	31
Marked enlargement	5
Extreme enlargement	1
Obstructive biliary cirrhosis	2
Hypertrophic biliary cirrhosis (infective)	5
Cirrhosis of the liver in children	2
*Cirrhosis with malaria	5
*Cirrhosis with kala-azar	4
*Syphilitic cirrhosis	2
Syndrome of Banti	
Marked enlargement	1
Moderate enlargement	1

* Included under the respective headings, shown here for comparison

If we are to analyse those cases of portal cirrhosis showing splenic enlargement, on the one side we have a group of 55 cases showing slight enlargement which could be regarded as being due to chronic venous congestion of the spleen from decompensation of the portal circulation. On the other hand we have 31 cases where the

enlargement is more than 500 g, five cases where the spleen is over 1,000 g and one case of extreme enlargement with a weight of over 1,500 g. These are cases where the splenic enlargement cannot obviously be accounted for on the basis of chronic venous congestion.

It is possible that these are cases where the spleen seems to bear the brunt of the toxæmia and the changes in the liver can only be regarded as coincident. There were cases that merged imperceptibly into the type described by Banti (1894) with marked thickening of the reticulum, widening of the sinuses, hyalinization of the trabeculae and endophlebitis of the splenic vein. These changes, however, I do not regard as something distinctive, but more or less similar to the changes that occur in portal cirrhosis. Durr (1924) who had an opportunity of examining Banti's sections is not inclined to regard the '*fibro-adenie*' of Banti as a specific process occurring only in this condition. The same changes may be met with in portal cirrhosis. Widening of the sinuses which is regarded as distinctive is also commonly met with in portal cirrhosis. The endophlebitis spreading to the portal vein is not constant. It is met with in malarial spleens. Early hyalinization of the malpighian vessels that Banti regards as the first stage of the whole process is met with in other conditions. In fact as Krumbhar (1927) puts it 'there is a discouraging lack of specificity in the lesions'. Nageli (quoted by Kaufman, 1929) claims that all the stages of Banti's syndrome were met with in lues and suggests that to the syndrome of Banti the ætiological diagnosis may be added such as malaria, syphilis, etc. Widening of the sinuses, and reticular fibrosis and endophlebitis have been met in a few of my cases in chronic malaria, but the condition here passes on to a more advanced stage with gradual disappearance of the adenoid structures, no doubt due to the irritative effect of the pigment. In this series of cases, there are two instances of Banti's syndrome where anæmia and splenic enlargement were followed by cirrhosis of the liver. It is curious that, apart from these cases, these records show no instances of splenic anæmia as such, one finds many instances where the splenic enlargement has not been put down to any definite cause. It looks as if these pathologists even in the nineties were content to regard splenic anæmia as a clinical syndrome without any uniform pathologic basis.

Cases of malaria showing cirrhotic changes in the liver amount only to five in our series. These are all grouped together under malaria. The existence of a malarial cirrhosis is a matter of acute controversy. Osler (1929) has never come across a single instance in 15 years. Rolleston and McNee (1929) doubt whether the cirrhosis occurring in malaria is only a coincidence. Rogers (1930) in Calcutta has never come across any case though he postulates an amoebic cirrhosis. Tirumurti and Radhakrishna Rao (1933), in Vizagapatam, have no instance to record. On the other hand the effect of malaria in inducing damage to the liver has been emphasized by Sinton and Hughes (1924). Hughes (1933) from Lahore puts forward the instance of a group of cases of cirrhosis, some of them running a course like Banti's syndrome, where the clinical history and the post-mortem evidence of malaria are striking. He argues that 'pathological changes in the portal spaces of the liver are similar to those in the spleen, viz, cellular hyperplasia followed by fibrosis'. Hughes describes a case where the gradual change could be followed from chronic malaria with enlargement of the liver and spleen to cirrhosis of the liver with ascites. From a pathologist's point of view, when fibrotic changes occur in the spleen following the chronic hyperplasia induced by the presence of parasites,

it seems unreasonable to deny that such changes can possibly occur in the liver. The demonstration of the reticulo-endothelial system by Aschoff (1924) has brought to us a clearer understanding of the relations between the liver and spleen, and Eppinger (1920) would have us define many diseases of the spleen as hepato-lienal diseases. Protozoal disease, where the importance of the reticulo-endothelial system is becoming more apparent, when it involves the spleen, is bound to have similar reactions on the liver.

When we pass on to the next group of cirrhosis with kala-azar, Rogers (1930) has drawn attention to similar cirrhotic changes. He has described an 'intracellular cirrhosis' in the cases of over two years' standing. Here, the early enlargement of the liver is due to a stuffing of the reticulo-endothelial system, a reticulo-endotheliosis, which has its counterpart in the spleen and the bone-marrow. Stimulation of this system is met with in protozoal diseases like malaria and kala-azar. There is ample evidence in the mononucleosis of the blood, and the enlargement of the spleen and the liver. Whether this is followed by a reactive fibrosis is a disputed point. Shanks and De (1932) have examined the liver and spleen in twenty-six consecutive cases of kala-azar and found fibrotic changes in the liver only in 30 per cent of cases, while in the spleen they found fibrotic changes only in 20 per cent of cases. They therefore consider that fibrosis of the spleen and cirrhosis of the liver are not regular features of kala-azar. I have tried to assess the proportion showing fibrotic changes in the spleen in our records. Out of our series of 155 cases of kala-azar, adhesive perisplenitis was met with in 21 cases while thickening of the capsule was met with in 36 cases making a total of 57 cases with evidence of fibrosis, or 36.7 per cent, a figure higher than that obtained by Shanks and De. It is thus clear that, while fibrotic changes are not common, they occur in a certain proportion of cases, the incidence probably depending on the chronicity of the disease. Maximow's (1933) studies on the potentialities of the reticulum cells have taught us that this undifferentiated reticular syncytium has got powers not only of forming the littoral cells which line the sinuses of the spleen, lymphatic glands, bone-marrow and the liver, but it is also capable of differentiation to reticulum fibres, which in turn become converted into collagen in pathological conditions. It is therefore clear that the hyperplastic reticulo-endothelium is capable of a grosser change into fibrous tissue provided the stimulus is chronic. There seems to be no valid reason to deny that fibrotic changes can occur in the liver and spleen in such cases, especially as there is definite post-mortem evidence of an association in a certain proportion of cases.

Of the two cases of cirrhosis in children, I am not at present able to say anything definite. Radhakrishna Rao (1933) in Vizagapatam has put forward the possibility of a syphilitic origin in such cases, but it appears to me that cirrhosis of childhood that is met with here may be possibly related to infantile cirrhosis which is so common. 'Toxic cirrhosis' is another factor to be reckoned with.

With regard to the five cases of biliary cirrhosis recorded, one is undoubtedly the result of inflammatory changes spreading from the gall-bladder and bile-ducts. The others are probably of an infective nature, though I have no definite evidence on this point. The two cases of obstructive biliary cirrhosis were associated with the presence of gall-stones.

There are four cases of syphilitic cirrhosis in adults, where the occurrence of irregular scars and gummata gave rise to the condition called '*hepar lobatum*'.

GROUP IV

Splenic enlargement associated with disease of the hæmatoporetic system
Total cases 24

Leukæmia—Total number of cases	9
Moderate enlargement	1
Marked enlargement	1
Extreme enlargement	7

In all these cases of leukæmia there was associated enlargement of the liver

Pernicious anæmia—There were seven cases of pernicious anæmia with splenic enlargement which was slight in five cases and moderate in two

Ancylostomiasis—Five cases of ancylostomiasis showed slight enlargement of the spleen which was not due to malaria. The spleen was soft and pale in colour and corresponded to the pale septic spleen met with in septicæmias. The nature of this enlargement is obscure, but the possibility of toxic absorption from the worm or from the lesions in the intestine has to be borne in mind

Unspecified type of anæmia—Slight enlargement was met with in three cases of unspecified types of anæmia, where there were no other factors to account for the condition

GROUP V

Chronic hyperplasias Total cases 388

1	Tuberculosis—Total number showing enlargement	84
	Simple enlargement	65
	Miliary tubercles	14
	Caseous tuberculosis	4
	Tuberculous infarction	1
	Slight enlargement	77
	Moderate enlargement	6
	Marked enlargement	1
2	Syphilis—Total number with enlargement	20
	Simple enlargement	14
	Gummata in spleen	6
3	Hodgkin's disease—Total number	2
4	Actinomycosis—Total number	1
5	Leprosy—Total number	2
6	Malaria—Total number	124
	Slight enlargement	69
	Moderate enlargement	45
	Marked enlargement	6
	Extreme enlargement	2
	Weight not recorded	2
7	Kala-azar—Total number	155
	Slight enlargement	37
	Moderate enlargement	73
	Marked enlargement	30
	Extreme enlargement	3
	Weight not recorded	12

Chronic hyperplasias form a well recognized group occurring most often in the infective granulomata and in protozoal diseases. There are 84 cases of tuberculosis, mostly fibro-caseous tuberculosis of the lungs with enlargement of the spleen. In most of these cases, the enlargement is of the nature of a simple hyperplasia of the littoral cells such as is met with in the septic spleens. Miliary tubercles occurred in only 14 cases. Caseous tuberculosis of the spleen was met with in four cases. In one of these cases, the enlargement of the spleen would appear to be the chief lesion, primary tuberculosis so called, and there was an old scar in the lung showing the mode of entrance of the bacillus. There was one case where typical wedge shaped infarctions, occurring in a case of tuberculosis, showed histologically the tuberculous nature of the changes affecting the vessel.

There were 20 cases of syphilis showing enlargement, and of these six showed gummata in the spleen. Two instances occurred of Hodgkin's disease, and one of actinomycosis, which was secondary to actinomycosis of the liver. Two cases of leprosy showing splenic enlargement are of interest.

There are 155 cases of kala-azar where a diagnosis was made on post-mortem findings. These cases date from the year 1903 when the parasite was first discovered by Donovan in a spleen smear. Subsequent records show 'the resistant bodies' of Donovan in the spleen smears. The disease was then looked upon as '*protoplasmosis*'. Donovan, however, emphasized the difference between these bodies and trypanosomes with which they were originally confused. There are a few records showing the fatal results of spleen puncture in its early days. The spleen in this disease often showed considerable enlargement, this enlargement was generally soft, the capsule was tense and stretched, the cut surface was bulging and 'glazed' and had a violet or reddish violet colour which is characteristic. Capsular changes, I have indicated, were present in a greater proportion of cases in Madras than in Bengal, probably owing to the greater chronicity of the types met with here. Histologically, the picture is one of hyperplasia of the littoral cells lining the sinuses which are stuffed with parasites. We have a typical instance of a disease affecting the reticulo-endothelial system, a reticulo-endotheliosis, the '*sinus reticulosis*' of Ross (1933) and other modern writers.

Enlargement due to malarial infection occurred in 124 cases. The appearance of the spleen was usually distinctive. With attacks of malarial fever sufficient to cause enlargement, the pulp became altered in colour from a dark brown to greyish brown, slaty grey, almost a dark grey, and sometimes tarry black. However, if one were to attempt to assess the incidence of malarial enlargement many factors would have to be considered. Malaria had been looked upon as the one cause of splenic enlargement in India, till kala-azar was recognized and separated as a distinct entity. The view is now gaining ground that types of splenic enlargement that are recognized in Europe may be equally common here and one is no longer content with a diagnosis of chronic malaria to explain these enlarged spleens. Divergent views exist, however, even to-day. Tirumurti and Radhakrishna Rao (1933) point out that though 'chronic malaria is diagnosed clinically very often in Vizagapatam, the same feature is not found in the autopsy records'. De gives an incidence of only 6.5 per cent from autopsy records in Calcutta. Hughes and Shrivastava (1931) report, however, a high incidence of malarial spleen in Lahore. The diagnosis is based on the history of repeated attacks of malaria. Hughes (1933) records the finding of malarial changes in the spleen, though parasites are not found in the

blood and no signs of active malaria are present. Connor (1933), in analysis of the figures from Bombay, Calcutta and Rangoon, postulates the existence of a group of postmalarial and possibly post-kala-azar types of splenomegaly, where malaria or kala-azar might be regarded as having only initiated changes in the spleen.

The following case is an instance of this type for which I am indebted to my colleague Dr C R Krishnaswamy of the Royapuram Hospital for permission to cite.

The patient, A L, a Hindu woman of about thirty years, was admitted to the Royapuram Hospital on 6th November 1933 for a chronically enlarged spleen with anaemia. She was a multipara had three children all healthy. There was no history of miscarriage. She had been getting profuse periods for the last four years. There had been enlargement of the abdomen and anaemia.

The patient denied a history of fever. There was no history of syphilis. She complained of weakness in the hands and legs and paresthesia. Examination revealed an enlarged hard, spleen reaching up to the level of the umbilicus. The liver was not enlarged and there was no enlargement of the lymphatic glands. There was marked pallor, slight puffiness of the face and slight pigmentation of the palms and soles. The heart was normal except for a soft systolic murmur at the pulmonary area. The lungs were healthy. The pupils were sluggish to light on both sides, the knee jerks were brisk and the plantar response normal. The haemoglobin was 55 per cent, R B C count 3,050,000, colour index 0.9, differential leucocyte count showed polymorphs 65 per cent, lymphocytes 15 per cent, monocytes 5 per cent, eosinophiles 15 per cent, basophiles 1 per cent, no malarial pigment in the leucocytes, no malarial parasites, fragility test-hemolysis complete 0.3 per cent almost complete 0.4 per cent, normal limits. van den Bergh reaction negative, direct and indirect Wassermann reaction strong positive. Kahn reaction positive. Spleen puncture was done by Dr C R Krishna swami on 13th November, 1933. Examination of the smears showed no parasites, but malarial pigment giving the reactions of hemozoin. The coagulation time was 6 minutes, stools showed hook worm ova and cysts of *E. coli*. Urine showed no abnormality.

In this case the demonstration of malarial pigment in the spleen smear is conclusive evidence of old malarial infection. The negative history of malarial fever and the absence of parasites in the blood indicate, either that the patient had suffered at one time from malaria, that the attacks were mild and not noticed, but that gradually this infection had died down and that some other contributory factor was responsible for the splenic enlargement, or it may be that the malaria was latent but smouldering, with parasitic multiplication too slight to produce evidence in the blood or affect the temperature chart. The strongly positive Wassermann and Kahn reactions, if taken as indicative of syphilitic infection, would supply the contributory factor.

To decide whether a splenic enlargement is really due to malaria, the pathologist has to go by definite criteria before these divergent views could be settled. The factors that have to be considered are (a) the morphology of the spleen especially with regard to colour changes of the pulp, (b) the occurrence of perisplenitis, (c) the microscopic demonstration of parasites or pigment in the spleen and liver, (d) histochemical tests by which this pigment could be recognized as hemozoin. It is true, malarial pigment can be distinguished from hemosiderin by its colour and by the absence of the prussian blue reaction. Brown granules of hematoidin are sometimes met with in the spleen and difficult to distinguish. Hemozoin however is dissolved by ammonium sulphide while blood pigments are blackened. Unstained sections should always be examined under the microscope with a drop of ammonium sulphide under a cover slip, when the gradual solution of the pigment can be watched. An examination of sections of the liver is also of equal importance in all cases of splenic enlargement—since the picture here is much less confusing—the littoral cells of von Kupffer being the phagocytes of malarial pigment.

GROUP VI

UNCOMMON TYPES OF SPLENIC ENLARGEMENT TOTAL CASES 47

A Chronic peritonitis, perihepatitis, perisplenitis

There were four cases of the Concato syndrome with extensive hyaloseritis involving the serous surfaces of the peritoneum, the mesentery, the intestines, the liver and the spleen. Slight splenic enlargement was present in all the four cases. Only one of these cases really corresponded to the polyserositis of Concato with involvement of the pleura, peritoneum and pericardium. In the other three the changes were confined to the peritoneal cavity. These corresponded to those cases of chronic superior peritonitis described by Sprawson (1921), the 'endemic ascites' of Megaw (1921), but there were no dysenteric lesions in the intestine. In one case of my own, where the lesions were critically studied, the ætiological factor was obscure. There was nothing to indicate antecedent syphilis or tuberculosis, there were no lesions in the stomach, intestine, appendix, or gall-bladder. The Wassermann reaction, however, was not tested.

B Enlargement associated with jaundice

There were four cases of well-defined enlargement associated with jaundice, the enlargement being moderate in three and marked in one.

C Enlargement with primary carcinoma of the liver.

The high incidence of primary carcinoma of the liver has been noted by Rogers (1925) in Calcutta and by Basu and Vasudevan (1929) in South India. The large proportion of splenic enlargement in these cases has hitherto been overlooked.

Out of 22 cases recorded 15 showed slight enlargement, six showed moderate enlargement and one case marked enlargement. Cappell (1923) has drawn attention to the presence of cell emboli in the spleen in many cases of tumour growth, secondary deposits however do not occur, as a rule, because the spleen has the function of destroying these cells. The splenic enlargement that was met with in primary carcinoma of the liver may be of a reactive nature similar to the condition met with in cirrhosis. The enlargement was generally slight, the pulp was rather firm, but distinctly hyperplastic, fibrotic changes being slight. Small soft white nodules were met with in the spleen in two of these cases, but histological details are wanting.

D Enlargement with other types of malignant disease

• One case of thyroid carcinoma showed slight enlargement, and the same feature was met with in two cases of epithelioma, one involving the tongue and the other the cheek. Primary carcinoma of the colon and of the rectum were responsible for two cases. Enlargement of the spleen secondary to lymphosarcoma is a well-known condition, the spleen being part of the hæmatopoietic system. We have two such instances on record, one being due to lymphosarcoma of the mesentery and the other of the intestine. One case of sarcoma of the kidney also showed enlargement. In none of these cases were secondary nodules met with. The only instance of

secondary growth was in a case of *mycosis fungoides* already reported (Basu, Bhaskara Menon and Pandalai, 1929). There were multiple tomato-like tumours on the skin and death had occurred as a result of visceralization.

E Enlargement due to hydatid disease

There are two instances in these records of enlargement due to hydatid disease. In both cases the enlargement was extreme the spleen weighing over 3,000 g. In both cases this was secondary to multiple hydatids in the peritoneal cavity.

F Enlargement due to follicle hyperplasia

Two cases are on record of this condition, which was first described by Brill, Baehr and Rosenthal (1925), as 'giant lymph follicle hyperplasia' and by subsequent writers as instances of 'reticulosus'. In these two cases on record the spleen showed slight enlargement. The cut surface showed numerous, white sago-like bodies scattered throughout the pulp. These did not give the staining reactions of amyloid natural and were enlarged lymph follicles. No description exists regarding the changes in the lymphatic glands. Histological details are meagre so that it is not possible to classify these any further. In one case congenital cystic kidneys were also present.

G Splenic enlargement with amyloid deposit

There are three instances of amyloidosis in two of which syphilis was the ætiological factor.

H Enlargement with lipid deposits

There is one recent instance in my personal series of lipid deposit in the spleen, which does not correspond to any of the well-known types described in Europe. The deposits occurred in a case of hæmangioma of the adrenal gland. This was a case of diabetes. The spleen was enlarged and there were two small irregular areas, each of the size of a small pea at the upper pole, one in the capsule, and the other in the pulp 0.5 cm. below. There were co-existing caseous foci in the spleen and other signs of tertiary syphilis. The nodules were of a yellow-ochre colour resembling in the appearance the cortical adenomas of the adrenal. A scar from the capsule passed down into the substance of the gland, and around this there were minute, yellow, irregular areas of the same deposit. Histologically, foamy degeneration of the cells with faint striations, such as is met with in the Gaucher type, was met with. The deposit was localized to these spots. Frozen sections showed that the lipid material was anisotropic and easily stained with Sudan III. Some of the littoral cells in the pulp also showed slight deposit, but the cells were not much enlarged. I take this as an instance of localized lipid storage in the spleen probably of a compensatory nature owing to the derangement of the adrenal cortex on the same side. The case is being reported elsewhere. Diabetic lipæmia, it is true, may give rise to generalized lipoidosis, but in this instance there was a localized deposit at the upper pole.

- KRUMBHAR, E B (1927)
 MAXIMOW, A A (1933)
 OSLER and McCRAE (1927)
 McNEE, J W (1932)
 MEGAW, J W (1921)
 OSLER, W (1929)
 RADHAKRISHNA RAO, M V (1933)
 ROGERS, L (1925)
 Idem (1930)
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 ROLLESTON, H, and McNEE J W (1929)
 ROSS, J (1933)
 SHANKS, B, and DE, M N (1932)
 SINTON J A, and HUGHES, T A (1924)
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 pp 507-08
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Jour Path Bact, **37**, No 2, p 311
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APPENDIX

SHOWING THE CLASSIFICATION OF THE VARIOUS CASES OF ENLARGEMENT
OF THE SPLEEN

TABLE I

Showing cases of enlargement of the spleen due to diseases of the hæmatoporetic system

SERIES I —Leukæmia

Post-mortem number	Date	Weight, oz	Cause of death
642	2-4-07	36	Liver 79 oz , rupture aneurysm
698	8-9-07	72	Myeloid leukæmia
271	12-5-11	64	Liver 64 oz , leukæmia
379	4-11-11	64	Leukæmia.
1328	5-4-19	74	Leukæmia , intraperitoneal hæmorrhage
	6-8-25	40	Myeloid leukæmia
	10-4-25	40	Myeloid leukæmia.
2010	30-9-26	158	Leukæmia , bronchopneumonia , liver 122 oz
2284	19-3-28	19	Myeloid leukæmia

Total number of cases 9

Extreme enlargement 7

Marked enlargement . 1

Moderate enlargement 1

SERIES II —Ancylostomiasis

Post-mortem number	Date	Weight, oz	Cause of death
111	6-8-07	9	Ancylostomiasis
196	5-10-09	17	Ancylostomiasis
714	22-8-13	8	Ancylostomiasis
1938	6-8-25	11	Ancylostomiasis
2080	28-3-27	10	Ancylostomiasis

Total number of cases 5

Slight enlargement 5

Enlargement due to malaria 2 (not included in this series)

SERIES III—Unspecified type of anæmia

Post-mortem number	Date	Weight, oz	Cause of death
17	24-2-95	9	Anæmia
236	28-11-04	12	Anæmia
781	13-2-14	8	Anæmia
Total number of cases			3

SERIES IV—Pernicious anæmia

Post-mortem number	Date	Weight, oz	Cause of death
667	27-7-07	18	Pernicious anæmia
1130	24-1-17	8	Pernicious anæmia
1143	3-1-18	11	Pernicious anæmia
1144	5-1-18	12	Pernicious anæmia
1180	4-5-18	20	Pernicious anæmia
1712	1-2-23	8	Pernicious anæmia
270	28-10-12	16	Pernicious anæmia
Total number of cases			7
Slight enlargement			5
Moderate enlargement			2

TABLE II

Showing cases of enlargement of spleen due to kala azar

Post-mortem number	Date	Weight of spleen	Cause of death	Capular changes
181	2-8-04	21	Kala-azar	Capsule thickened
182	3-8-04	39	Kala-azar, pneumonia	Capsule thickened
196	2-9-04	15½	Kala-azar	Perisplenitis
201	7-9-04	22	Kala-azar	Perisplenitis
243	9-12-04	40	Kala-azar, pneumonia	Capsule thickened
455	8-7-05	10	Kala-azar, carcinoma of stomach	
459	15-3-06	15	Kala-azar (peripneumonia)	Capsule thickened
463	23-3-06	Not known	Kala-azar	Perisplenitis
469	10-4-06	17	Kala-azar	Perisplenitis
470	10-4-06	17	Kala-azar	
	14-4-06	36	Kala-azar dysentery	Capsule thickened
	4-5-06	37	Kala-azar, dysentery	
	19-5-06	37	Kala-azar dysentery	Perisplenitis
	25-5-06	20	Kala-azar, dysentery	
	19-8-06	10	Kala-azar	
	25-8-06	10	Kala-azar	
	23-9-06	43	Kala-azar dysentery	
	10-10-06	52	Kala-azar dysentery	
	24-1-07	25	Kala-azar	
618	2-2-07			
640	22-3-07	9	Ancylostomiasis	
6180	5-10-09	17	Ancylostomiasis	
714	22-8-13	8	Ancylostomiasis	
1938	6-8-25	11	Ancylostomiasis	
2080	28-"	10	Ancylostomiasis	

5

5

alaria 2 (not included in this series)

TABLE II—*contd*

Post-mortem number	Date	Weight, oz	Cause of death	Capsular changes, other lesions
696	30-8-07	24	Kala azar, dysentery	Perisplenitis Perisplenitis
722	4-11-07	30	Kala azar, dysentery	
730		38	Kala azar, dysentery	
742	7-1-08	13	Kala azar	
759	8-2-08	20	Kala azar, dysentery	
770	23-2-08	Not known	Kala azar, dysentery	
	14-4-08	19	Kala azar	
	25-5-08	28	Kala azar	
	8-8-08	18	Kala azar	
	19-8-08	22	Kala azar, nephritis	
80	24-8-08	24	Kala azar, dysentery	Capsule thickened
371	19-9-08	25	Kala azar, pneumonia	
876	20-9-08	33	Kala azar, dysentery	
888	12-10-08	13	Kala azar	
898	22-10-08	17	Kala azar, bronchopneumonia	
914	8-11-08	34	Kala azar, dysentery	
924	19-11-08	24	Kala azar, dysentery	
929	29-11-08	32	Kala azar, pneumonia	
951	29-12-08	23	Kala azar, bronchopneumonia	
22	1-2-09	18	Kala azar	
29	13-2-09	34	Kala azar	
61	22-3-09	14	Kala azar, myocardial degeneration	
68	16-4-09	31	Kala azar, cancerum oris	
75	7-5-09	48	Kala azar, nephritis	
76	7-5-09	34	Kala azar	
120	30-6-09	18	Kala azar	
136	17-7-09	29	Kala azar, dysentery	

TABLE II—*contd*

Post-mortem number	Date	Weight, oz	Cause of death	Capsular changes, other lesions
137	18-7-09	16	Kala-azar, dysentery	
142	27-7-09	12	Kala azar, amœbic dysentery	
144	29-7-09	11	Kala azar, pneumonia	
157	12-8-09	17	Kala azar, perisplenitis	
204	20-10-09	34	Kala azar, lobar pneumonia	
215	5-10-09	26	Kala-azar	
218	8-11-09	18	Kala-azar, lobar pneumonia	
28	8-2-10	33	Kala azar, lobar pneumonia	
79	29-4-10	18	Kala azar, acute yellow atrophy	White infarct
83	6-5-10	48	Kala azar, dysentery	
106	4-7-10	12	Kala azar, dysentery	
114	18-7-10	48	Kala-azar	
138	24-8-10	52	Kala-azar	Liver 81 oz
153 ³	20-9-10	46	Kala azar, pneumonia	Capsule thickened
158	23-9-10	36	Kala azar	Liver 79 oz, irregular cirrhosis
163	27-9-10	16	Kala azar, amœbic dysentery	
165	4-10-10	80	Kala-azar, pneumonia	
166	5-10-10	24	Kala azar, heart failure, pneumonia	Capsule thickened
186	14-11-10	26	Kala azar, dysentery	
325	8-8-11	16	Kala-azar, dysentery	
347	19-9-11	52	Kala azar, dysentery	
348	21-9-11	30	Kala azar, dysentery	
434	22-1-12	42	Kala azar	Liver 64 oz
441	2-2-12	16	Kala azar	
453	12-2-12	Not known	Kala azar	
457	20-2-12	Not known	Kala azar, bacillary dysentery	
474	6-3-12	16	Kala azar, dysentery	

TABLE II—*contd*

Post-mortem number	Date	Weight, oz	Cause of death	Capsular changes, other lesions
480	12-3-12	17	Kala azar	Perisplenitis
378	29-10-11	24	Kala azar	
389	13-11-11	23	Kala azar, bronchopneumonia	
399	23-11-11	18	Kala azar	
404	2-12-11	26	Kala azar, pneumonia	
519	29-5-12	20	Kala azar	
540	29-7-12	20	Kala azar, dysentery	
560	21-8-12	18	Kala azar, dysentery	
588	19-10-12	11	Kala azar, cancerum oris	
689	1-1-13	29	Kala azar	Liver 56 oz
611	5-12-12	24	Kala azar	Liver 48 oz, cirrhosis, perisplenitis
644	3-2-13	23	Kala azar	Liver 54 oz
660	25-3-13	24	Kala azar	
665	31-3-13	22	Kala azar, anaemia	Perisplenitis
788	3-3-14	35	Kala azar, pneumonia	Capsule thickened
797	17-3-14	18	Kala azar, bronchopneumonia	Old infarcts
808	31-3-14	56	Kala azar, bronchopneumonia	
810	3-4-14	18	Kala azar	
699	15-7-13	32	Kala azar	
701	24-7-13	16	Kala azar	
703	26-7-13	40	Kala azar	
706	30-7-13	32	Kala azar	
707	5-8-13	24	Kala azar	
715	25-8-13	12	Kala azar, cancerum oris	
777	10-2-14	28	Kala azar	
841	22-7-14	48	Kala azar, bronchopneumonia	Capsule thickened
855	22-8-14	33½	Kala azar, bronchopneumonia	Capsule thickened
882	2-11-14	26	Kala azar, bronchopneumonia	Capsule thickened

TABLE II—*concl'd*

Post-mortem number	Date	Weight, oz	Cause of death	Capsular changes, other lesions
892	10-12-14	28	Kala azar, necrosis of jaw	
906	19-1-15	37	Kala-azar, pneumonia	
908	22-1-15	22	Kala-azar	
933	16-4-15	46	Kala azar, tuberculosis	Perisplenitis
957	16-9-15	15½	Kala azar	
973	1-11-15	24	Kala azar, lobar pneumonia	Perisplenitis
991	2-2-16	24	Kala azar	
1001	2-3-16	17	Kala azar, cancerum oris	
1061	15-3-17	46	Kala azar, pneumonia	Capsule thickened
1067	5-5-17	21	Kala azar, anæmia	
1100	17-8-17	30	Kala azar pneumonia	Capsule thickened.
1155	5-2-18	10	Kala azar	
1208	15-7-18	25	Kala azar	
1261	19-10-18	40	Kala-azar	Infarcts, cirrhosis, smear positive, capsule thickened
1289	14-12-18	41	Kala azar	
1651	30-8-22	37	Kala azar, cancerum oris	
1746	16-5-23	16	Kala azar	
1767	20-8-23	48	Kala azar	
1771	31-8-23	21	Kala-azar	
1777	22-9-23	56	Kala azar	
1813	28-7-24	30	Kala azar	Capsule thickened
1840	4-9-24	22	Kala azar	
1900	3-3-25	8	Kala azar	
2236	20-12-27	20	Kala-azar, bronchopneumonia	Perisplenitis
2334	20-12-27	20	Kala azar	Capsule thickened
2609	14-12-29	24	Kala-azar	

This table shows only 135 cases of Kala azar from the Madras General Hospital, twenty cases from the Government Royapuram Hospital are not shown here

TABLE III

Showing enlargement of the spleen due to syphilis

Serial No	Post-mortem number	Date	Weight, oz	Cause of death
1	191	13-8-32	11	Syphilis, gumma spleen
2	172	20-7-98	16	Syphilis, tertiary
3	277	9-12-09	11	Syphilis
4	242	8-12-04	8	Syphilitic cirrhosis, infarct
5	941	11-12-08	10	Syphilis, gumma liver
6	32	21-2-08	8	Syphilis, gumma heart
7	120	26-7-10	10	Syphilis, aneurysm of aorta
8	101	23-6-10	14	Syphilitic peritonitis
9	199	30-8-09	26	Gumma liver, dysentery
10	259	5-4-11	21	Syphilis, gumma liver
11	6	8-1-10	12	Syphilis, aneurysm of aorta
12	478	11-3-12	16	Syphilitic cirrhosis
13	479	5-3-12	12	Gumma spleen, gumma brain
14	657	18-3-13	7½	Syphilitic cirrhosis, liver
15	836	27-5-14	14	Syphilis, gumma spleen
16	992	2-2-16	8	Syphilis, gumma heart
17	1004	20-3-16	14	Gumma spleen, early syphilitic cirrhosis
18	1503	22-2-21	8	Syphilis
19	2057	13-2-27		Gumma spleen, gumma liver
20	678	10-5-13	12	Syphilis, gumma of spleen, aneurysm

Total number of cases	20
Slight enlargement	17
Marked enlargement	2
Weight not known	1

TABLE IV

Showing splenic enlargement due to primary carcinoma of the liver

Serial No	Post mortem number	Date	Weight, oz	Description
1	46	23-12-89	24	
2	35	21-5-94	8½	
3	79	19-12-24	15	
4	93	18-12-95	10	
5	160	11-6-98	17	
6	34	26-2-09	44	
7	39	7-3-09	12	
8	234	14-12-09	16	
9	222	12-1-11	8	
10	787	2-3-14	21	
11	619	15-12-12	18	
12	794	14-3-14	14	
13	668	4-4-13	16	
14	806	27-3-14	11	
15	733	27-11-13	9	
16	1637	10-8-22	17	
17	1856	15-10-26	8	
18	2007	30-9-26	20	
19	2009	9-3-27	12	
20	2337	30-6-28	24	
21	2362	13-8-28	16	
22	2401	9-10-28	24	

Total number of cases	22
Slight enlargement	15
Moderate enlargement	6
Marked enlargement	1

TABLE V

Showing splenic enlargement of indefinite causation The attempted classification of these cases is shown

Serial No	Post-mortem number	Date	Weight, oz	Appearances of spleen	Cause of death and complications
SERIES I—(Unrecognized kala-azar)					
1	10	20-2-94	36	Capsule slightly thickened	? Kala azar (unrecognized)
2	40	26-6-94	37	Capsule thickened	? Kala azar, cancrum oris (unrecognized)
3	18	28-2-95	24	Perisplenitis	? Kala azar (unrecognized), pneumonia
4	26	16-4-95	24		? Kala azar (unrecognized), pneumonia
5	7	4-2-96	24		? Kala azar (unrecognized), pneumonia
6	62	31-12-96	26		? Kala azar (unrecognized), liver 96 oz
7	179	14-9-98	38		? Kala azar (unrecognized), ulcer oesophagus
8	275	2-4-02	27	No malariae parasite	? Kala azar
9	282	13-11-02	33	Adherent capsule	? Kala azar (unrecognized)
10	276	2-11-02	27	Thickened capsule	? Kala azar (unrecognized), cancrum oris
11	272	31-10-02	37	Perisplenitis	? Kala azar (bacillary dysentery)
12	332	7-4-03	40		? Kala azar (unrecognized)
13	336	24-4-03	40		? Kala azar (unrecognized)
14	20	27-8-03	46 1/2	Perisplenitis	? Kala azar (unrecognized), liver 70 oz
SERIES II					
1	223	15-1-11	34	Enlarged, soft	Oedema larynx, liver 74 oz, ? kala azar, no parasite found
2	403	3-12-11	32	Soft, chocolate	Pneumonia, liver 56 oz, ? kala azar
3	523	11-6-12	24	Decomposed no parasite or pigment	Liver 83 oz, ? kala azar

TABLE V—*contd*

Serial No	Post mortem number	Date	Weight, oz	Appearances of spleen	Cause of death and complications
SERIES II— <i>contd</i>					
4	540	22-7-12	20	Soft, no L D or pigment	Liver 64 oz, ? kala azar
5	581	7-10-12	23	No pigment or para sites	Liver 54 oz, ? kala azar
SERIES III					
1	461	24-2-12	68	Fibrosed, capsule thick	Irregular cirrhosis, no parasite or pigment, liver 68 oz
2	960	18-9-15	81	Perisplenitis, infarct	Early cirrhosis, no parasite or pigment, liver 84 oz
3	356	30-9-11	72	Soft	Slight cirrhosis, pneumonia, liver 64 oz
SERIES IV					
1	81	27-12-93	104	Gray, pink	Pulmonary infarction, liver 120 oz, leukæmia
SERIES V					
1	48	19-9-93	46	Soft, dark	? Malaria—Acute peritonitis, arthritis, endocarditis and pericarditis
2	756	1-2-08	28	Dark, fibrosed	? Malaria, rabies
SERIES VI					
1	1081	18-6-17	30	Soft	Peritonitis, abscess near spleen
SERIES VII					
1	912	6-11-08	24	Perisplenitis, infarct	Anæmia, ? splenic anæmia

TABLE V—concl'd

Serial No	Post mortem number	Date	Weight, oz	Appearances of spleen	Cause of death and complications
SERIES VIII					
1		7-7-21	34	Soft, no fibrosis, perisplenitis	Acute meningitis
2	79	14-11-22	30	Soft, no fibrosis	Chronic pulmonary tuler culosis
3	154	14-8-31	33	Soft, no fibrosis	Chronic pulmonary tuber culosis
4	644	15-4-07	32	Soft, no fibrosis, perisplenitis	Chronic pulmonary tuber culosis
5	238	17-8-02	40	Soft, no fibrosis	Liver 72 oz, peritonitis, amœbic dysentery, ? post kala azar, bronchopneumonia
6	94	1-6-09	32	Firm, fibrosed	Bronchopneumonia
7	194	3-10-09	38	Soft, capsule thick smears negative	Acute nephritis, gangrenous appendicitis
8	439	30-1-12	26	Fibrosed	Liver 70 oz, cerebral abscess, pneumonia
9	663	27-3-13	20	Perisplenitis, no pigmentation	Dysentery
10	784	21-12-14	31	No pigmentation	Amœbic dysentery
11	1321	10-3-19	44	Chocolate colour, perisplenitis	Liver 60 oz, lobar pneumonia
12	1406	6-1-20	40	Chocolate colour, perisplenitis	Mitral stenosis
13	1492	27-12-20	36	Soft	Lobar pneumonia
14	1618	6-7-22	67	Firm, no pigment	Liver 84 oz, perisplenitis lobar pneumonia
15	1831	25-8-24	38	Fibrosed, no pigment perisplenitis	Lobar pneumonia
16	1932	11-9-25	36	Soft, no fibrosis	Bronchopneumonia
17	2026	28-11-26	32	Firm	Tuberculous arthritis
18	2515	23-5-29	30	Firm	Amœbic dysentery

Total number of cases	45
Moderate enlargement	25
Marked enlargement	15
Extreme enlargement	5

RAT-FLEA SURVEY OF PEERMADE DISTRICT, TRAVANCORE

BY

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PEERMADE DISTRICT in Travancore State forms part of the southern range of the Western Ghats and comprises a hilly country. In a large part of the district the elevation is 2,000 to 3,000 feet above sea-level, sometimes even more. To the east of the district there is a precipitous fall into the Cumbum Valley in Madras Presidency, while on the western side the level falls less abruptly. The district extends from latitude $9^{\circ}25' N$ to $9^{\circ}49' N$ and from longitude $76^{\circ}44' E$ to $77^{\circ}25' E$ and covers an area of about 450 square miles, part of which is reserve forest. The cleared area is planted with tea and rubber and to a smaller extent with cardamom and coffee. The population is sparse and consists chiefly of labour engaged on the plantations.

Peermade district could be divided into two topographical regions, (a) a high-lying region on the east which forms the larger part of the district with an elevation usually more than 2,000 feet, and (b) a western region comprising the foot-hills and the lower area having an elevation ranging from 1,200 feet to 200 feet above sea-level. A line drawn north-west to south-east from a point about two miles to the west of Peermade would divide the district into the two respective regions.

It would appear that there have been sporadic cases of rat plague and possibly also of human plague in Peermade district for some years past, but much attention was not bestowed on the question till 1932 when several cases of human plague occurred in some estates in the district. Control measures were then started and at the same time, a rat-flea survey of the entire district was taken up with a view to determine the rat-flea fauna of the area. The survey started in September 1932 and was concluded in March 1933. Data in regard to the following points were collected: (1) incidence of rodents in association with man and human dwellings, (2) flea fauna parasitic on these

rodents, and (3) the distribution and incidence of the different species of rat fleas. The results of the survey are reported in the present article.

PROCEDURE

Rat-traps were set in human habitations in the afternoons and collected again on the following morning. Traps containing captured rats were enclosed in cloth bags to prevent fleas escaping during transit and were brought to a temporary field laboratory. After removing the cloth bag, the rat was dropped into a large glass-jar and then killed with chloroform. Any stray fleas that happened to be sticking to the cloth bag or to the trap were caught in tubes. The dead rat after identification was combed and brushed over a table covered with white cloth and finally shaken and tapped on the table to dislodge any fleas that might still be sticking to it. The fleas brushed off the rat as well as those found sticking to the walls of the glass-jar were put into specimen tubes with spirit and kept for determination. The traps were then washed and aired and the baits renewed before they were set again. Generally from 100 to 200 traps were laid in each locality.

Wooden traps were used during this survey and they were found to be more effective than wire traps. An advantage with this type of rat-trap is that it ensures single rat catches which are desirable in flea survey work. When more than one rat are caught in a trap, as it often happens in wire traps, there is the likelihood of fleas quitting the hosts during the disturbance caused by rats fighting with one another. A further advantage with the wooden trap is that as the interior of the trap is dark, the fleas do not leave the host so readily as they may do in wire traps.

THE SURVEY.

The survey covered only the inhabited area of Peermade district while forest areas were omitted. One hundred and thirteen localities were visited and the total number of traps set was 22,471. Two thousand three hundred and sixty-four rats were caught of which 2,356 were *Rattus rattus*, 4 *Bandicota*, 3 *Mus* and 1 *Pachyura**. *R. rattus* was the principal rodent associated with human dwellings in the district.

The fleas collected from these rats and identified numbered 5,925. The following nine species of fleas were observed during the survey: *Xenopsylla cheopis* Roths, *X. astra* Roths, *X. brasiliensis* Baker, *Stivalus ahala* Roths, *Ctenocephalus felis orientis* Jordan, *Echidnophaga gallinaceus* Westw., *Leptopsylla himalaica* Roths, *Ceratophyllus nilgiriensis* J and R, and *Pulex irritans* Linn.

The relative incidence of the different species of fleas and their distribution in relation to the respective hosts are furnished in Table I.

**Pachyura*, commonly known as the 'musk rat' is not a rodent.

TABLE I

	Number of rats	Total fleas	<i>X cheopis</i>	<i>X astia</i>	<i>X brasiliensis</i>	<i>Stivalius</i>	<i>Ctenocephalus</i>	<i>Felidnophaga</i>	<i>Leptopsylla</i>	<i>Ceratophyllus</i>	<i>Pulex</i>
<i>P rattus</i>	2,356	5,696	3,047	1,492	48	552	6	541	2	7	1
<i>Bandicota</i>	4	23	1	22	1						
<i>Mus</i>	3	1				1					
<i>Pachyura</i>	1	0									
Man		205					77				128
TOTALS	2,364	5,925	3,048	1,514	48	553	83	541	2	7	129

From the point of view of richness of flea parasites, *R rattus* seemed to be the principal host of rat fleas in Peermade district. *Bandicota* had a higher average flea index than *R rattus* but it was comparatively rare in the district. *Xenopsylla cheopis* and *X astia* were the commonest rat fleas. *Ctenocephalus* and *Pulex* were found only in small numbers on *R rattus* but it seems likely that the actual incidence of these two species is much higher than the catches off *R rattus* indicate. It would appear that they are fairly common in many localities in Peermade high-lying area, but as they spend most of the time away from the host and do not ordinarily stay on the host longer than it is necessary to have a feed of blood, they are not represented in any large numbers in collections off *R rattus*. They not uncommonly attack man and could be collected by stepping over dirt and debris in dark corners of rooms when they get on to the legs of a person. By this method the writer obtained 205 fleas (77 *Ctenocephalus* and 128 *Pulex*) from a single house in Periyar estate.

SEX PROPORTION

Table II furnishes the relative incidence of males and females of the different species of rat fleas collected in Peermade district.

TABLE II

Species	Total	MALES		FEMALES	
		Number	Percentage	Number	Percentage
<i>X cheopis</i>	3,048	1,697	55.7	1,351	44.3
<i>X astia</i>	1,514	732	48.3	782	51.7
<i>X brasiliensis</i>	48	29	60.4	19	39.6
<i>Stivalius ahalæ</i>	553	264	47.7	289	52.3
<i>Ctenocephalus felis orientis</i>	83	23	27.7	60	72.3
<i>Echidnophaga gallinaceus</i>	541	40	7.4	501	92.6
<i>Leptopsylla himalaica</i>	2	1	50.0	1	50.0
<i>Ceratophyllus nilgiriensis</i>	7	4	57.1	3	42.9
<i>Pulex irritans</i>	129	50	38.8	79	61.2

In regard to five species, namely, *Xenopsylla cheopis*, *X astia*, *Stivalius ahalæ*, *Ceratophyllus nilgiriensis* and *Leptopsylla himalaica*, there was no marked disproportion in the incidence of the sexes. There was, however, some disproportion in *X brasiliensis* in which 60.4 per cent were males. In regard to *Ctenocephalus*, the males occurred in small numbers as compared with the females. The males were 27.7 per cent and the excess of females over males was 44.6 per cent. A similar disproportion was observed in *Pulex irritans*, the males being 38.8 per cent of the total. The disproportion between the number of males and females was most striking in the case of *Echidnophaga gallinaceus*. In this species, the males were only 7.4 per cent while the incidence of females was disproportionately high, namely 92.6 per cent.

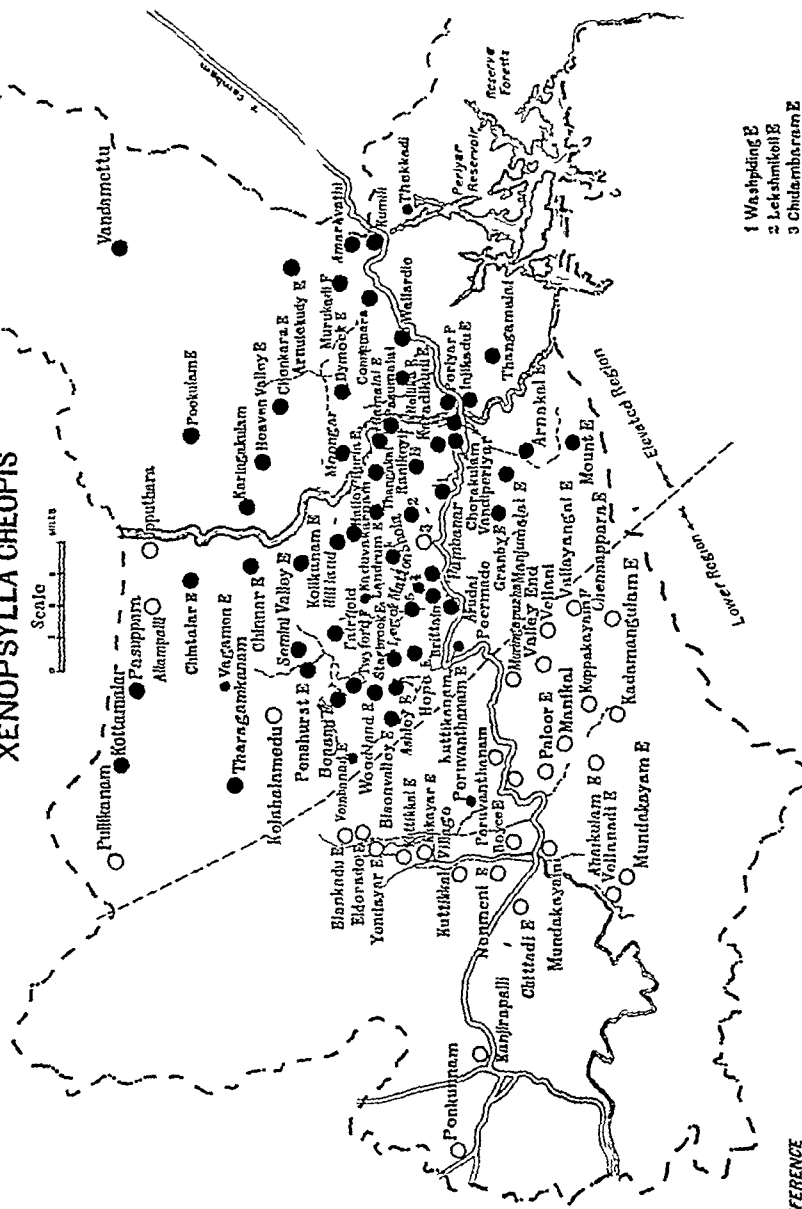
PEERMADE HIGH-LYING REGION

It was mentioned that Peermade district could be divided into two distinct topographical regions, a high-lying region on the east and a lower one on the west. The former region is hilly, has an elevation generally more than 2,200 feet and has a temperate and a comparatively less humid climate. The lower region on the west is generally less than 1,200 feet in elevation and has a warmer and more humid climate than the eastern area. A line drawn in a N-W to S-E direction from near Murinjapula would divide the area into the two respective regions (see Map).

Peermade high-lying region extends from Kuttikanam on the west to Kumili, the eastern end of the district. The area has an average annual rainfall of 200 inches at the western boundary and 65 inches on the east.

MAP OF PEERMADE DISTRICT SHOWING DISTRIBUTION OF XENOPSYLLA CHEOPIS

Scale
0 1 2 miles



REFERENCE

- Localities with cheopis index 1.0 and above
- Localities less than 1.0
- Localities where X. cheopis were absent

- 1 Washpiding E
- 2 Lekshimikoll E
- 3 Chidambaram E
- 4 Pambanar E
- 5 Western & Glenmary E

Seventy-seven localities were surveyed in this region. The rat incidence varied considerably and in some localities it was found to be as high as 30 per 100 traps set. The average rat incidence based on the rat catch was 9.5 per 100 traps set. The total number of rat fleas collected was 4,423. Table III shows the relative incidence of the different species of fleas as represented in these collections —

TABLE III

Species	<i>X. cheopis</i>	<i>X. astia</i>	<i>X. brasiliensis</i>	<i>Stralulus</i>	<i>Ctenocephalatus</i>	<i>Echinophagus</i>	<i>Leptopsylla</i>	<i>Ceratophyllus</i>	<i>Pulex</i>
Number	3 047	71	7	540	79	541	2	7	129
Percentage	68.9	1.6	0.2	12.2	1.8	12.2	0.1	0.2	2.9

Xenopsylla cheopis comprised a high proportion (69 per cent) of fleas collected in the Peermade high-lying region. *Stralulus* and *Echinophagus* formed 12.2 per cent each. *X. astia* was poorly represented and formed only 1.6 per cent. Other species were comparatively rare. An interesting feature of the flea fauna of this region is the occurrence of hill species like *Ceratophyllus nilgiriensis* and *Leptopsylla himalaica* and of temperate species like *Pulex irritans*. In India the last mentioned species is generally confined to hilly areas.

The flea index of localities investigated varied between 0 and 10 (average 2.9). The general flea index is not as important as the specific flea data, namely, incidence of the transmitter species. The two important data are (1) the *X. cheopis* rate (percentage of *X. cheopis* to total fleas) and (2) the *R. rattus cheopis* index (average number of *X. cheopis* per rat). Of the two, the latter is the more valuable one as the *cheopis* rate may often give a wrong impression. For instance preponderance of fleas not concerned with plague transmission may unduly lower the *cheopis* rate of a locality although the incidence of *X. cheopis* per rat may be high. In a similar manner, sparseness of other fleas may raise the *cheopis* rate. In epidemiological studies, the *Rattus rattus cheopis* index furnishes the more reliable information in regard to the transmitter factor in plague.

The *cheopis* rates of the 77 localities investigated in Peermade high-lying area varied from 0 to 100 and averaged 72.4 per cent. The frequency distribution of localities with different *cheopis* rates is shown in Table IV —

TABLE IV

<i>Cheopis</i> rate	0-10	10-30	30-50	50-70	70-90	Over 90
Number of localities	9	5	6	8	20	29
Percentage	11.7	6.5	7.8	10.4	26.0	37.7

In 74 per cent of the localities, the *cheopsis* rate was higher than 50. In 16 out of 77 localities, the *cheopsis* rate was 100. The results show that in Peermade high lying region *cheopsis* rates are very high.

Table V furnishes the frequency distribution of the *R. rattus cheopsis* index in the localities surveyed —

TABLE V

<i>Cheopsis</i> index	0	0.1-0.5	0.6-1.0	1.1-1.5	1.6-2.0	2.1-3.0	3.1-4.0	4.1-6.0	Over 6.0
Number of localities	8	4	7	14	11	16	12	4	1
Percentage	10.4	5.2	9.1	18.2	14.3	20.8	15.6	5.2	1.3

In a large proportion of the localities investigated the *cheopsis* index was high. In 75 per cent of the localities, the *cheopsis* index was higher than 1.0 and in 43 per cent, it was above 2.0. The highest *cheopsis* index recorded here was 10.0. Hirst (1927) considers that a *R. rattus cheopsis* index of 1.0 would seem compatible with the continuous spread of epizootic plague during the favourable season. From that point of view, 60 localities out of 77 investigated in the Peermade high-lying area have the requisite density of *X. cheopsis* to cause epizootic plague.

PEERMADE LOWER REGION

The western region of Peermade has an elevation ranging from 1,200 feet to 200 feet. The average annual rainfall is high (160 inches to 200 inches). Thirty-six localities were surveyed in this region. Seven thousand four hundred and three traps were set and 929 rats (*R. rattus*) were caught. The average rat incidence is 12.5 per 100 traps set. The total number of fleas collected and identified was 1,502. The following five species were observed in this area: *Xenopsylla cheopsis*, *X. astia*, *X. brasiliensis*, *Stivalvus ahalæ* and *Ctenocephalus felis orientis*.

Table VI shows the relative incidence of the different species of rat fleas observed in this region.

TABLE VI

Species	<i>X. cheopsis</i>	<i>X. astia</i>	<i>X. brasiliensis</i>	<i>Stivalvus</i>	<i>Ctenocephalus</i>
Number	1	1,443	41	13	4
Percentage	0.1	96.1	2.7	0.9	0.6

Xenopsylla astia formed the bulk of the fleas collected here (96 per cent of the total). An interesting feature of this area was the absence of four genera of fleas which were observed in the high-lying region, namely, *Leptopsylla*, *Ceratophyllus*, *Pulex* and *Echidnophaga*. Even more striking was the almost total absence of *X. cheopis*, a species that was found to be abundantly common in the higher area. Only one specimen out of a total of 1,502 fleas examined from the lower region was found to be of this species.

The general flea index in this area varied between 0 and 4, averaging 1.6. In 35 localities out of 36 investigated in the lower region *X. cheopis* was absent and in one locality, namely Peruvanthanam estate, a solitary specimen of *X. cheopis* was collected. The survey showed that *X. cheopis* was practically absent in the Peermade lower region. In regard to the high-lying area, it was mentioned that 60 localities out of 77 investigated had the requisite density of *X. cheopis* to cause epizootic plague. In the Peermade lower area, none of the localities investigated had the sufficient number or density of *X. cheopis* to cause epizootic plague.

FLEA FAUNA OF THE TWO REGIONS

The contrast in the flea fauna of the two regions in Peermade district was indeed very striking. In the lower region, cold-climate forms like *Ceratophyllus*, *Leptopsylla* and *Pulex* were absent. *Stivalius ahalæ*, which is a hill species, occurred to the extent of 12.2 per cent in the high-lying region, whereas in the lower region it was poorly represented (0.9 per cent). This species was common at elevations above 2,500 feet and its incidence decreased rapidly as one proceeded to lower levels. It was found in small numbers at 1,000 feet and below that level it was practically absent. *Xenopsylla brasiliensis* had a larger prevalence (2.7 per cent) in the lower region than in the elevated region, where it formed only 0.2 per cent of the total.

The findings in regard to the incidence of *Xenopsylla cheopis* and *X. astia* in the two areas are of interest. In the high-lying region, *X. cheopis* occurred extensively and formed 68.9 per cent of the total catch, while *X. astia* was observed in small numbers (1.6 per cent). In the lower region on the other hand *X. cheopis* was almost entirely absent and 96.1 per cent of the fleas were *X. astia*. As Hirst (1927) says 'X. astia and X. cheopis appear to be adapted biologically to distinctly different sets of climatic conditions', it seemed probable that climatic conditions were responsible for the variation in the incidence of *X. astia* and of *X. cheopis* in the two regions. *X. astia* is evidently not well adapted to the temperate conditions prevailing in the elevated area, and the marked decrease in the incidence of this flea in the high-lying area is probably largely due to this factor.

SUMMARY

Peermade district could be divided into two distinct areas having different flea fauna —

1. A high-lying zone with fleas of the temperate climate like *Ceratophyllus*, *Pulex*, *Stivalius*, and *Leptopsylla*, a high incidence of *Xenopsylla cheopis* and a comparative sparsity of *X. astia*.

2 A lower zone where temperate species were absent, *X cheopis* was very rare and the bulk of the flea population consisted of *X astia*. No marked difference was noticeable in regard to the nature of the population, type of habitation, or species of rat found, which could explain this difference in the flea fauna.

The writer is indebted to Dr. Karl Jordan for his kindness in determining the specimens of *Stivalius*, *Ceratophyllus* and *Leptopsylla* that were sent to him.

REFERENCE

HIRST, L. F. (1927)

Ceylon Jour. Sci., Section D, **1**, Part 5 30th April

SENSITIZATION AND ANTI-BODY PRODUCTION IN GRANULOMA GENITO-INGUINALE

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THE present investigation was conducted with a view to determine whether there is any tissue sensitization or anti-body production in *granuloma genito-inguinale* (*Syn-granuloma venereum* or *granuloma inguinale*). Originally it was started with the hope that a specific skin test could be worked out which would find application in the early detection of this distressing disease with its frequent devastating sequelæ. It is true that established cases do not give much trouble to the clinician, but it is equally true that in places where the disease is endemic, cases are invariably missed in their early stages owing to the existence of a variety of other ulcerative conditions of the genitalia without there being any differentiating features between them. This disease is not of uncommon incidence in the Circars of the Madras Presidency as can be inferred from the statistics of the King George Hospital, Vizagapatam. Seventy-five cases, all from the northern districts of the province and most of them in an advanced stage, were admitted into this hospital during the past three years. These experiments to work out a simple diagnostic test led to a further examination for the presence of any complement-fixing substance in the blood of the patient, specific to this disease. This latter investigation was undertaken with a purely academic interest.

Source of material for the antigen—The causative agent of the disease is yet unknown and therefore any specific protein that may be sharpening the cell reactivity to it can not be directly obtained. Passing mention may be made in this connection of the tendency of many observers to incriminate the Donovan bodies, constantly associated with the disease, as its specific cause. Presence within the tissue cells of organisms other than the ætiologic is by no means an uncommon finding in stained smears of pathological discharges or tissue juices. Sometimes the intracellular bodies associated with *granuloma genito-inguinale* do appear like the

inclusion bodies described in certain virus diseases, and one is accordingly tempted to consider whether a virus ætiology is not likely in this disease. It is assumed here that the infecting agent, whatever it be, resides in the tissues involved in the ulcerative process. Tissue shavings from the edge of the ulcers from typical cases and showing the Donovan bodies were utilized throughout these studies as material from which to prepare the antigen. The sores were cleaned by antiseptic washing and kept as much free from surface contamination as possible with antiseptic dressings for two days, after which pieces of tissue were removed aseptically into a sterile petri dish to be utilized as the source of antigen.

Cutaneous test -- The tissue thus obtained was mixed with an approximately equal volume of sterile sand, well rubbed up to a paste in a sterile mortar and then transferred to a test tube of suitable size. To this was added sterile normal saline in the proportion of 1 c c to a gramme of the tissue, mixing them thoroughly by the vigorous shaking of the tube for about half a minute. Complete sterilization was then effected by heating it in the water-bath at 56°C for one hour on three consecutive days. It was subsequently tested for sterility and kept in the ice-chest as the stock antigen.

One-tenth c c of the clear slightly opalescent supernatant fluid was injected intradermally on the anterior aspect of the forearm of the patient. Control injections on individuals not suffering from the disease were also likewise given. Both the cases as well as some of the control series showed varying grades of reaction, but in the former it was always much more pronounced and of a severer type. Control experiments with non-granulomatous tissue extract were not done, and it is possible that the mild reactions present in some of the normal cases, especially when the antigen was comparatively unripe, were due to the action of certain histamine-like substances present in all tissues particularly the skin and known to be able to produce non-specific skin reactions in many individuals.

The reaction reached its peak in four to six hours and remained as such for an equal interval slowly subsiding thereafter. It consists of a swelling at the site of inoculation associated with redness, induration and pain locally. A few of the patients also complained of some tenderness and pain in the axillary lymphatic glands and slight constitutional disturbance. The pain disappeared in a couple of days and the swelling and the induration in another two days. Cases under varying stages of treatment as well as completely healed ones gave a positive response (see Table).

TABLE

Serial number	Duration of the disease	Time of doing the test in relation to treatment	Result of the test
1	3 weeks	Before	+
2	2½ months	"	+
3	11 "	During	+
4	2 years	After	+

TABLE—*concl'd*

Serial number	Duration of the disease	Time of doing the test in relation to treatment	Result of the test
5	1½ years	Recurrent case, before	+
6	3 months	During	+
7	1 month		+
8	1 "	,	+
9	1 year		Doubtful
10	7 months	Before	+
11	5 years	Recurrent case before	+
12	9 months	During	+
13	8 "	"	+
14	1 month	Before	+
15	6 months	During	+
16	2 years		+
17	2 months	Before	+
18			+
19	15 years	During	+
20	20 "	During and after healing	+ on both occasions
21	2 weeks	Before	+
22	2 months	"	+
23	1 year	"	+
24		2 years after healing	+
25	3 years		+

Twenty-five cases were tested in all during a period of six months and with the same antigen. Within limits, the older the antigen the less non-specific it appeared to be as inferred from the observation that the resulting reaction on controls was almost negligible as the antigen became older. Although the results in this series are not very definite, still there seems to be scope for the development of a diagnostic intradermal test for this disease by the employment of antigens prepared by improved methods. The sifting out of early cases of granuloma genito-inguinale is by no means easy, and the usefulness of the test, if one is satisfactorily evolved, would be in overcoming this difficulty. A modified antigen prepared for the

complement-fixation test was also tried as the cutaneous antigen with negative results. The above experiments tend to demonstrate that the causative agent, whether protozoal, bacterial, or of a virus nature, does sensitize the body, enhancing the cell reactivity whatever the degree of such a sensitization be.

Complement-fixation test—Three types of antigen prepared from the same source were employed in the test, an alcoholic extract prepared as follows and reinforced with cholesterol as in the case of the Wassermann antigen. —A weighed quantity of tissue obtained as mentioned above was mixed with sand in a mortar and well ground up. Alcohol was added to it to the extent of 10 c.c. to every gramme of the unmixed material and the contents of the mortar were transferred to a stoppered bottle. It was kept at incubator temperature for three days, the bottle being vigorously shaken as often as possible during this period. Next it was kept in the ice-chest for a fortnight, at the end of which period the emulsion was filtered and the filtrate preserved in the ice-chest. At the time of the test cholesterol was added in the same proportion as in the case of the Wassermann antigen, diluting the mixture thereafter with physiological saline as necessary. The next type of antigen tested was a saline suspension of the deposit left after the evaporation of the pure alcoholic extract. The third was the saline extract used for the intradermal test. Dilutions of 1 in 1, 1 in 2, 1 in 4, 1 in 8, 1 in 16, and 1 in 32 were employed in the test. None of the antigens used evinced any anti-complementary behaviour in these dilutions. The usual Wassermann technique was followed in the conduct of the test and readings were taken two to three hours after its completion.

In all, eleven specimens were tested in this way at different times including many normal sera as controls. Though all the specimens tested were from frank typical cases only, there was no inhibition of hæmolysis at any time except once when there was a partial fixation of the complement in the case of one specimen which was also strongly positive for Wassermann reaction. The explanation of this last point is too obvious for any comment. In all other cases the complement was not fixed. It might be argued that the tissue extracts used had not any antigen value, but the occurrence of the positive skin test lends support to the assumption that the ætiologic agent exists locally in the lesions, and, in the absence of any certain knowledge about the nature of the offender, the method detailed in this communication seems to be the only way of acquiring any sort of antigen. From the above it is not unreasonable to conclude that the infecting agent in *granuloma genito-inguinale* does not stimulate the production of any specific complement fixing immune body.

CONCLUSION

Employing extracts of tissue obtained from the ulcers of *granuloma genito-inguinale* the evidence for the occurrence of allergic sensitization of the cells was not unfavourable, while that for the presence of any specific complement-fixing antibody in the blood was negative.

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A COMPARISON OF MACCONKEY'S BILE-SALT BROTH AND DOMINICK-LAUTER BROTH IN ROUTINE WATER ANALYSIS

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THE very large number of false presumptive positive tests that are encountered in routine water analysis, is a serious problem mainly for American water bacteriologists

Thus, Frost (1906), Creel (1914) and Hall and Ellefson (1918) found a large number of spurious presumptive positives in water analysis due to sporulating lactose-fermenting anærobes Levine (1920) employing American technique reported similar findings in his work in France Raab (1923) found that anærobes were responsible for 94 per cent of the positive presumptives in Minneapolis water Meader and Bliss (1923) isolated a number of anærobes from positive lactose broth cultures

Spore-forming ærobes producing false presumptive positive tests have been found in water by A Meyer (1909), E M Meyer (1918), Ewing (1919), and Sohn (1924)

False presumptives due to symbiotic action have been recorded by Sears and Putnam (1923) Sohn (1924), Leitch (1925) and Dunham, McCrady and Jordan (1925) have demonstrated that gas production, when due to anærobes, is the result of a symbiotic relationship

Janzig and Montank (1928), in dealing with filtered and chlorinated water samples, were able to obtain in 1927 confirmation in *only seven* out of 1,878 presumptive positive tubes (i e , 0.4 per cent) and in 1928 in *not one* of 915 similar presumptive positives

Leahy (1930) found that positive presumptive tests for *B. coli* on the Rochester City water between 1928 and 1929 failed to confirm in 51.5 per cent of the samples tested and in 1930 the failure was 100 per cent.

In their attempts to minimize or eliminate this trouble which involved time and labour in confirmatory technique, American bacteriologists have tried and advocated the use of various dyes and alterations in pH values of the enrichment media. In fact, much of the recent American literature on water bacteriology deals with this aspect of the problem.

Most of the American bacteriologists have accepted the standard lactose broth as the best medium for preliminary enrichment and subsequent sub-cultural confirmation in water analysis. Jackson (1906), Hale (1916), and Levine (1922), however, have advocated the use of bile as an inhibitory agent in the enrichment medium. The absence of bile or any other inhibitory agent in the standard lactose broth of the American standard methods accounts, in our opinion, for the very large number of spurious positive tests. They are due largely to the aerobic and anaerobic, sporulating and non-sporulating lactose fermenters present in most waters and also possibly to bacterial synergism and symbiosis (Dominick and Lauter, 1929). These phenomena have been studied at great length by various workers all over the globe. Ruchhoff *et al* (1931), in their exhaustive study of water bacteriology, referred to the spurious tests due to these factors and advocated a purification method.

They were probably dealing with cultures isolated from the standard lactose broth (devoid of bile). It is our belief that, if they had employed preliminary enrichment on a lactose bile medium such as MacConkey's (containing 1.5 per cent bile-salt), the results would have been different.

Dominick and Lauter (1929) published the results of their successful use of a new medium, which was designed to eliminate the false positive presumptives in routine water analysis. They claimed certain very definite advantages for this medium, viz. —

(i) by using methylene blue and brom cresol purple in the new medium in certain dilutions, a definite determination can be reached as to the presence or absence of *B. coli* in any water at the end of 24 hours, *without* the necessity for any further confirmation on solid media,

(ii) in proper proportions, the two dyes give a direct colour change (from blue through green to yellow) that is produced much more rapidly than the formation of gas in standard lactose broth and with equally accurate results,

(iii) the new medium has been used in their laboratories as a direct confirmation procedure on all waters with parallel series of standard lactose broth cultures on all filtered and chlorinated waters. It has given excellent results with a complete elimination of positives due to symbiotic lactose-splitting combinations and other spore formers,

(iv) in a series of readings taken after incubation at 37°C it was found that *B. coli* strains were able to reach a pH of 5.6 and 5.7 consistently, while strains of *B. aerogenes* were unable to reach below 6.8, showing that *coli* has the faculty of utilizing the sugar present at pH 7.1 to a greater degree than *aerogenes*, and that the buffer action of the phosphate salts present tends to produce an environment that is

more favourable to earlier acid and gas production by *coli*, and insufficient carbohydrate for fermentation by the other members of the family

Since the publication of the above findings, several other workers have also reported on the use of this medium Leahy, Freeman and Katsampes (1931) tested 1,116 samples from 358 different sources of water-supply, using the two media They found that the total number of samples showing gas, and the percentage of tubes showing gas which confirmed, were much higher with the new medium than with the standard lactose broth The percentage confirmation for standard lactose broth tubes showing gas in 24 hours was higher than for tubes showing gas in 48 hours, whereas the percentage confirmation with the Dominick-Lauter tubes showing gas in 24 and 48 hours was approximately the same The percentage confirmation for lactose broth increased as pollution of the water under test increased, whereas the percentage confirmation for the new medium remained approximately the same Of the total number of samples showing a positive presumptive test 100 per cent of those from the new medium and only 68.7 per cent from standard lactose broth confirmed The new medium was thus found to be far superior to the standard lactose broth (without bile) for the detection of *B. coli* in water

McCants (1931) reports very favourably on the new medium and considers it to be quick, reliable and superior to the standard lactose broth He would, however, like more work done before final adoption of the new medium

Howard (1932) made a comparison of the standard broth with both the Dominick-Lauter and the brilliant green bile medium, he found that the brilliant green bile produced 1.4 per cent more confirmed results than lactose broth and Dominick-Lauter broth gave 0.7 per cent more than lactose broth in the case of raw water samples In filtered water samples, the advantage in the matter of confirmation appeared to lie with the lactose broth In chlorinated water, using lactose broth, three 24 and twenty-six 48 hours tubes showed fermentation out of which only one was confirmed, i.e., 3.4 per cent Brilliant green bile and Dominick-Lauter each had only one presumptive positive in 24 and one in 48 hours of which only one, i.e., 50 per cent, confirmed in each medium Therefore lactose broth would appear to show no advantage over Dominick-Lauter broth in dealing with raw and chlorinated water In unchlorinated filtered water the standard lactose broth showed a *coli* recovery rate of 2.4 per cent greater than the other two Howard concludes by saying that the success of the Dominick-Lauter medium depends on the use of dyes of established suitability, without which the medium may be of but limited value

Stewart (1933) refers to the work on this medium carried out at Arkansas by Jackson, Hale and himself and concludes that the medium is very promising as they have had excellent results in the comparatively small number of samples tested

Nolte and Kramer (1933) examined daily 17 samples of settled, applied, filtered and chlorinated finished water by the standard method and by the Dominick-Lauter procedure Seven hundred and sixty-nine samples were thus studied They found (a) a close correlation between the results obtained by the two methods on polluted river water, (b) a superiority for the new medium in the case of unfinished waters,

and (c) a definite superiority for the new medium in the case of finished waters. The superiority referred to, lay in the fact that by the Dominick-Lauter procedure a smaller percentage of presumptive positives was obtained, with a larger percentage of confirmations, than in the standard method procedure. They concluded that the Dominick-Lauter method as a routine procedure in their laboratory was the best, as being the simplest, quickest and most reliable.

Before proceeding to describe our work, it should be stated that the standard methods of the A P H A have *not* been adopted by us, either for preliminary enrichment or for subsequent confirmations. We consider them to be defective in more respects than one. For example, the use of lactose broth without any inhibitory agent would result in many false presumptives. The confirmation of the presumptive positives by morphological and staining characteristics *alone*, selecting *only two* colonies, suspected to be *B. coli*, from the solid medium and passing them again through the same lactose broth—without any inhibitory agent—appears to us, particularly in the light of Ruchhoff's elaborate purification studies, to be in the nature of a very speculative venture. Again, this process of confirmation gives no differentiation between *coli*, *aerogenes* or the *intermediates*—a distinction which most authorities consider important, except perhaps in the case of fully treated waters. We therefore make no apology for giving in some detail the methods adopted in this study.

The sample of water under test is added to MacConkey's lactose bile salt neutral-red broth and to the new medium, the strength and the quantities of water being shown in the following tables —

Details of cultures in MacConkey's medium (as adopted by Clemesha in India in 1908)

Number of dilution	Strength of medium	Amount of medium in c c	Water added in c c	Number of tubes
A	6 per cent peptone (Difco)	7	20	1
	1.5 per cent sodi taurocholate			
	1.5 per cent lactose (pure)			
	Neutral red			
	Reaction Alkaline pH 7.2 to 7.4			
B	50 per cent of A	7	10	2
C	50 " "	5	5	3
D	33 " "	3	1, 0 1, 0 0 1	3 of each

Details of cultures in Dominick-Lauter medium

Number of dilution	Strength of Dominick Lauter medium	Amount of medium in c c	Water added in c c	Number of tubes
A	Lactose 10 g Bacto peptone (Difco) 10 g K ₂ H PO ₄ 3H O 14.3 g KH PO ₄ 2 g Distilled water 2 litres Dissolve at 50°C to 60°C Add 16 per cent alc sol brom cres purple 4 c c 10 per cent aq sol erythrosin 4 c c 10 per cent aq sol methylene blue 4 c c	15	10	1 to 3
B	, " " ,	10	5	1
C	Dilute in ratio 600 c c dist water (neutral) to 1 litre broth A	5	10 01 001	1 1 1

The MacConkey and the Dominick-Lauter tubes are incubated at 37°C examined for acid and gas after 24 and again after 48 hours. A record is also made of the change of colour in the Dominick-Lauter broth tubes after these periods. At the end of the first 24 hours, the tube with the second smallest amount of water showing acid and gas is selected in each case (or the tube with the largest amount of water if no other tube is positive or if none of the tubes are positive). Dilutions from these tubes are sub-cultured on MacConkey's rebiplagar for discrete pink colonies, six of which are picked off after 24 hours' incubation into MacConkey's broth (D) tubes. After 24 hours' incubation, these cultures are tested in the following media, viz,

Nutrient or tryptophane broth for indole and motility, dipotassium hydrogen phosphate glucose broth for M R and V P tests, Koser's citrate solution, and the following sugars—saccharose, dulcitol, adonite and inulin.

The sugar fermentations are recorded after 48 hours, motility and Gram staining reaction after 18 to 24 hours, indole production after 24 hours if in tryptophane broth or after 72 hours if in nutrient broth. The M R and V P tests are carried out after four days. Koser's citrate test is read off after 24, 48 and 72 hours. The six colonies from each sub-culture have thus been classified as *coli*, *aerogenes* or *intermediates*. We claim that this gives a more convincing confirmation of the presumptive positives with each of the two media.

We have also attempted to group our samples according to their source and the results of the tests are tabulated accordingly. The gas readings at 24 and at 48 hours have been separately compared so as to allow full scope for a comparison of the two media.

We have thus examined over 70 samples drawn from different sources scattered over the whole of the Madras Presidency, which comprises an area of 143,870 square miles with a coast-line of over 1,700 miles. The annual rainfall varies from 150 inches on the west coast to about 24 inches on the east. Our samples may therefore be considered to be representative of different climatic and geological conditions, and to be exceptionally well fitted for making a comparison of the value of the two media in respect of different types of water. The American workers are more or less limited to a few types of water in their comparative studies. In most cases, we find that the results and conclusions relate to samples from a single filtration plant.

There was no conscious selection made of the samples in our series. Samples which arrived (packed in ice) in the course of the routine work of the laboratory between certain dates, were examined by the two procedures and the results recorded.

TABLE I

Source	Number of samples	MACCONKEY'S HALF SALT BROTH		DOMINICK LAUTER BROTH	
		Positive presumptives	Confirmed	Positive presumptives	Confirmed
A Wells	17	15	15	15	15
B Rivers	13	13	13	13	13
C Infiltration galleries	6	5	5	3	3
D Lakes and storage reservoirs	17	17	17	14	13
E Filtered (slow sand and rapid mechanical) water	12	8	7	10	7
F Chlorinated (all types)	7	1	1	3	3
TOTALS OF ALL SAMPLES	72	59	58	58	51

Table I gives the total number of samples examined, the number that yielded positive presumptive tests by the two methods, and the number of these positive that confirmed on the application of the tests described above.

(a) *Presumptive*—It will be seen that no definite superiority can be claimed for the new medium over MacConkey's broth. The new medium was, if anything,

inferior to the other in the case of D and only slightly better in E and F. The vast majority of the water-supplies in this province, and in fact in India as a whole, being derived from sources A to D, the use of the new medium appears to offer no special advantages over the time honoured and well tried MacConkey's medium.

(b) *Confirmatory tests*—In Table I, we have followed the American definition of 'confirmed tests' and include under 'confirmed' all samples which showed the presence of organisms which grew as pink colonies on MacConkey's agar and which were Gram negative, non-sporing and produced acid and gas in MacConkey's bile-salt lactose broth on secondary culture. *B coli*, *B aerogenes* and *intermediate* forms are all therefore included in the group. On this basis, while 98.3 per cent of the presumptive positives in MacConkey's broth confirmed for *colon-aerogenes*, only 93.1 per cent confirmed from the new medium. Again, 80.6 per cent of the total number of samples in the MacConkey's and 75 per cent in the new medium yielded members of the *colon-aerogenes* group.

TABLE II

A comparison of Dominick-Lauter medium with MacConkey medium on the basis of delicacy of reactions

Source	Number	DOMINICK LAUTER MEDIUM					
		More delicate		Equal to		Less delicate	
		24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
A Wells	17	3	3	7	12	7	2
B Rivers	13	0	1	4	6	9	6
C Galleries	6	1	1	3	3	2	2
D Lakes etc	17	3	6	4	7	10	4
E Filtered waters	12	5	7	6	11	1	4
F Chlorinated waters	7	3	3	4	4		
TOTALS	72	15	21	28	33	29	18
Percentage		20.8	29.2	38.9	45.8	40.3	25.0

In recording positive presumptive tests, reliance was placed on the production of acid and gas in MacConkey's broth tubes and on the production of gas together

with a direct colour change from blue to yellow in the new medium, in 24 and 48 hours. Table II shows that the new medium was more delicate than MacConkey's in 20.8 per cent of the samples after 24 hours' incubation, i.e., lactose fermenters were detected in smaller quantities of the inoculum in the new medium than in MacConkey's. In 38.9 per cent of the samples, both media yielded identical results. In the remaining 40.3 per cent of the samples, the advantage lay with MacConkey's bile-salt broth, the new medium being less delicate in detecting lactose fermenters.

The gas readings after 48 hours' incubation show that the new medium has gained under the first two heads. Even so, MacConkey's medium is found to have detected *colon-aerogenes* in smaller volumes than in the new medium in 25 per cent of the samples submitted to the two tests. As one of the claims made for the new medium is that a definite determination can be reached as to the presence or absence of *coli* (they include *aerogenes* and *intermediates* also under this term) in any water at the end of 24 hours, without the necessity of any further confirmation on solid media, the results after 48 hours' incubation are of very limited value.

Tables III and IV may be read together. The results of our fully confirmed tests have been reclassified in these two tables on the basis of two different sets of reactions used largely in differentiating *coli*, *aerogenes* and *intermediates*. The indole and methyl-red tests alone have been used in Table III. Koser's citrate utilization test together with the indole test has formed the basis of Table IV. In our hands there has been a remarkably perfect correlation between these two tests and we would therefore place greater reliance on this combination than on any other set or sets of tests.

Table III—68.7 per cent of the total number of organisms isolated from MacConkey's broth cultures and only 44.6 per cent of those from the new medium were true *B. coli*.

Table IV—Practically identical results for true *B. coli* have been obtained by this classification. The figures for *B. aerogenes*, however, show a difference, but the relative proportion has not been materially altered. The increase under this head is due to the fact that the *intermediates* (under the previous table) are assignable to one or the other of the two main groups (*B. coli* or *B. aerogenes*) by this classification. We would here like to emphasize the very definite advantage gained by including the citrate and indole tests in the routine procedure for confirmatory work especially in the tropical countries. This view is now held by English bacteriologists also (Topley and Wilson, 1930).

All the four, viz., indole, M.R., V and P, and Koser's citrate utilization tests, have been included in framing Table V. Although sixteen combinations are possible with the four tests, all our strains fitted into one or other of the nine given in the table. Following Ruchhoft *et al.* (1931) we have classified these nine combinations into three major groups, *coli*, *aerogenes* and *intermediates*. Combinations 1 and 2 form the true *B. coli*, combinations 3, 4, 5, 7 and 8 comprise the *intermediates* and 9 and 10 the *aerogenes*. It will be seen that the MacConkey's broth cultures have yielded on confirmation a larger proportion of true *B. coli* than the new medium. Two hundred and thirty-three out of three hundred and forty-two (68.1 per cent) from MacConkey's and only 136 out of 300 (45.3 per cent) from the new medium were *B. coli*.

TABLE III

A comparison of the lactose fermenters isolated from the two media on the basis of the M R and indole tests only

Source	Number of samples	MacCONKEY'S MILE SALT BROTH				DOMINICK LAUTER BROTH			
		Number of lactose fermenters	Coli M R + Ind +	Aerogenes M R - Ind -	Intermediates M R ± Ind ±	Number of lactose fermenters	Coli M R + Ind +	Aerogenes M R - Ind -	Intermediates M R ± Ind ±
A Wells	17	87	49	17	21	81	28	36	19
B Rivers	13	70	46	18	12	77	24	37	16
C Galleries	6	30	27	1	2	16	13	1	2
D Lakes, etc	17	102	78	17	7	69	39	23	7
E Filtered water	12	37	26	7	4	42	20	22	0
F Chlorinated	7	6	6	0	0	18	12	6	0
TOTALS	72	338	232	60	46	305	136	125	44
Percentage			68.7	17.7	13.6		44.6	41.0	14.4

TABLE IV

A comparison of the lactose fermenters isolated from the two media on the basis of the indole and Koser's citrate tests

Source	Number of samples	MACCONKEY'S BILE SALT BROTH				DOMINICK LAUTER BROTH			
		Number of lactose fermenters	Coli K-I+	Aerogenes K+I-	Intermediates K+I+	Number of lactose fermenters	Coli K-I+	Aerogenes K+I-	Intermediates K+I+
A Wells	17	87	48	33	6	83	32	48	3
B Rivers	13	76	44	29	3	77	25	48	4
C Galleries	6	30	27	3	0	16	8	8	0
D Lakes, etc	17	102	76	24	2	69	35	34	0
E Filtered water	12	37	26	11	0	42	19	23	0
F Chlorinated	7	6	6	0	0	18	11	6	1
TOTALS	72	338	227	100	11	305	130	167	8
Percentage			67.1	29.6	3.3		42.6	51.8	2.6

TABLE V
Classification based on indole, M R, V and P, and citrate tests together
The tests are noted in the table in the above order

Enrichment procedure	True coli		Intermediates					True aerogenes	
	1	2	+	+	+	+	+	9	10
MacConkey's broth	5	228	3	1	0	20	15	54	10
	68 1		13 2					18 7	
Dommick Lauter broth	4	132	2	1	1	27	12	115	6
	15 3		14 3					40 3	
TOTALS	9	360	5	2	1	53	27	169	16
Percentage	57 5		13 7					28 8	

Taking the V and P, and Koser's citrate tests alone, and considering that all organisms positive to both tests were *aerogenes* the new medium yielded 42.3 per cent while only 20.7 per cent of those from MacConkey's bile-salt broth were of this type

TABLE VI

Source	Number of samples	NUMBER SHOWING THE PRESENCE OF TRUE <i>B. coli</i> ON THE BASIS OF			
		KOSER'S CITRATE —	INDOLE +	METHYL RED +	INDOLE +
		MacConkey's broth	Dominick Lauter's medium	MacConkey's broth	Dominick Lauter's medium
A Wells	17	12	8	12	8
B Rivers	13	12	9	12	10
C Galleries	6	5	2	5	3
D Lakes and storage reservoirs	17	16	9	15	9
E Filtered water	12	5	5	5	5
F Chlorinated	7	1	2	1	2
TOTALS	72	51	35	50	37
Percentage		70.5	48.6	69.4	51.4

It will be seen from Table VI that preliminary enrichment in MacConkey's broth revealed the presence of true *B. coli* in about 70 per cent of the samples by either set of tests, while enrichment on the new medium showed them to be present in only about 50 per cent of the samples. By the use of the new medium alone therefore, about 20 per cent of the samples which contained true *B. coli* would have been passed.

As the results obtained by us did not appear to justify the claims made by the sponsors of the new medium, under the conditions of our first series of tests, it was considered desirable to obtain a small supply of the dyes used by them to see if the suitability of the dyes played an important part in the success of the medium as Howard (1932) had pointed out. Messrs. Dominick and Lauter kindly supplied a small quantity of their standardized dehydrated M. B., B. C. P. medium, prepared by the Difco Laboratories, as also a small quantity of the dye stocked by Eastman Kodak Company of New York. We made comparative tests with the new medium (i) as made originally by us with the brands of dyes available in our laboratory, (ii) as prepared from the dehydrated Difco product. Cultures in MacConkey's

broth served as controls. The results showed that the Difco dehydrated medium was slightly superior to our M B, B C P medium but not to the MacConkey's broth. Thus, while true *B coli* were isolated from 12 out of 19 samples in MacConkey's broth cultures, the new medium (ours and Difco) revealed their presence in 6 and 8 samples respectively. With the Difco product there was also a sharper colour change and a larger volume of gas in a shorter time. We therefore consider that variations in the results obtained with different brands of dyes constitute a distinct limitation to the usefulness of any medium.

There is one other point to which we wish to draw attention. The original article of Dominick and Lauter would appear to suggest that their M B, B C P medium was specific for *B coli* as against *B aerogenes*, and that the medium was specially suited for detecting true *B coli* in water. Our findings have failed to establish this claim, but on the other hand the use of this medium has resulted in the isolation of more *B aerogenes* by the fully confirmed tests. The sponsors of this method, according to a private communication (Dominick and Lauter, 1933), have apparently withdrawn their claim for specificity as they have stated that this new medium 'will inhibit only the spurious lactose fermenters outside of the *colon-aerogenes* group and inhibit not at all, members of the latter group'.

We may be permitted to state therefore that the new medium may be definitely superior to the standard lactose broth of the A P H A methods (6th Ed.) but it is definitely inferior to MacConkey's bile-salt broth in water analysis work. The fact that more samples showing true *B coli* are detectable by the MacConkey's bile-salt broth than by the new medium is sufficient proof of the greater usefulness of the former in routine water analysis. We have used this medium in this laboratory with uniformly satisfactory results for nearly a quarter of a century and false presumptive positives have never troubled us. The quality of the water as judged by the results of our tests has nearly always correlated with the quality expected from a knowledge of the local conditions.

We desire to express our grateful thanks to Lieut.-Colonel H H King, I M S, Director, and to Major W J Webster, M C, I M S, Assistant Director, of the King Institute, Guindy, for their valuable advice and helpful criticism during the course of our work and in writing up this paper.

SUMMARY AND CONCLUSION

Parallel tests on seventy-two samples of water, from different sources, have been carried out using MacConkey's bile-salt lactose broth and Dominick-Lauter methylene blue brom-cresol-purple broth respectively as the enrichment and selective media. Comparison indicates that the MacConkey broth test is more sensitive and more reliable.

Both as a selective medium for the presumptive positive test for *coli*, and as an enrichment medium to be followed up by sub-culture for the study of discrete colonies, and classification into *coli*, *aerogenes* and *intermediates*, MacConkey's broth (1.5 per cent bile) retains its position as the most satisfactory medium in water bacteriology.

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STUDIES ON THE ANTIGENIC STRUCTURE OF *VIBRIO CHOLERÆ*

Part VI

ANALYSES OF VIBRIO PROTEINS RACEMIZATION

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As a part of our research into the comparative structure of the cholera and cholera-like vibrios, we have undertaken the study of the protein constituents. The results obtained from the van Slyke analyses have already been reported (Linton, Mitra and Shrivastava, 1934). In this paper we are presenting a study of the effect of racemization on a series of vibrio proteins. In the 'Discussion' is included a resumé of the relationships among the three vibrio groups which we have recognized from our previous work (Linton and Shrivastava, 1933a, 1933b, and 1933c).

The method we have used depends essentially upon the observations of Kossel (1912) and Dakin (1912) that proteins dissolved in weak alkaline solutions gradually alter their optical properties. This change takes the form of a diminution of rotatory power, which is rapid at first, but gradually becomes less marked until after some days a constant value, much less than the original, is reached. This change in the rotatory power has usually been described as due to a racemization, although it is generally recognized that the term is incorrect. It appears probable that the variation in optical activity is due to an intra-molecular change, which results in the production of optically inactive amino-acids, rather than that equal quantities of *d*- and *l*-forms are produced, as in true racemization (Jordan-Lloyd, 1926)

In the earlier use of the method, the proteins were allowed to stand in alkali until their rotation had become constant. They were then hydrolysed in acid, the resulting amino-acids isolated, and their optical properties determined. The amino-acids, with the exception of glycine, are ordinarily optically active after acid hydrolysis. The amino-acids from the alkali-treated proteins were found to vary in this property. Some were optically active, some inactive and others partially active. If the same amino-acids in two proteins were found to have different optical properties it was evident that they could not have occupied similar positions in the original molecules, since they had been acted upon differently by the alkali. It is probable that intra-molecular changes occur only in the amino-acids which are completely within the protein molecule, and not in those which occupy terminal positions.

A number of proteins were studied in this way by the earlier workers. As Woodman (1921) has pointed out, the method is dependable but technically not entirely satisfactory. In the first place, the isolation and purification of the individual amino-acids is a lengthy and tedious process and one requiring a large amount of material. Secondly, the conclusion that one protein is distinct from another can be drawn only when the optical activity of at least one of its amino-acids is clearly different from that of the corresponding amino-acid of the other protein. Woodman showed that a much simpler method, based on the same phenomenon, could be used. His modification consisted in following the optical activity of the protein during the period when it was dissolved in alkali, and plotting the specific rotation against time. The curve obtained in this way was perfectly smooth, and had a concordant shape in duplicate experiments. He also found that two proteins of the same structure varied equally when the concentration of alkali used as a solvent was varied, as would be expected from the considerations on which the method is based.

Woodman showed that it was possible by this simplified method to establish the identity or non-identity of related proteins. By comparing the rate of racemization he found that the globulins of cow and ox sera and of colostrum were the same, while lactalbumin and serum albumin were distinct. The albumin of milk was found identical with the albumin of colostrum. He also found that the corresponding proteins of cow and ox sera were identical.

Since our previous work had shown that the proteins of the cholera and the cholera-like vibrios were closely similar as far as the van Slyke analyses could go, it seemed possible to apply the method outlined above as a more thorough test of their identity or non-identity.

METHOD OF PREPARATION OF THE PURIFIED VIBRIO PROTEINS

It was essential for the success of the comparative method to treat the proteins in the same way throughout the entire process of preparation and during the polarimetric readings. This cardinal point was kept in mind during the whole work.

The vibrios were grown on 5 per cent agar for 48 hours. The papain-digest broth in the agar contained 1 per cent of oxidizable matter. The heavy growth was washed off in 0.5 per cent phenol, allowed to stand at room temperature for a few hours and then washed repeatedly in the Sharples supercentrifuge until a 30 c c

or 40 c c portion of the wash-water did not reduce Benedict's solution after hydrolysis. Our previous experience had shown that any agar which had dissolved out during the incubation period would be removed by this treatment. The washed bacterial mass was dried in the air oven at 50°C.

After collecting about 40 grammes of dried bacterial substance, which represented the growth of approximately 350 Roux flasks, the purification was begun. This was somewhat modified by the exigencies of the material from Woodman's method, and was briefly as follows. The protein was finely powdered and taken up in about 2 litres of distilled water, and sufficient sodium hydroxide added to make a 1 per cent solution. The solution was centrifuged free from insoluble matter and acetic acid added until the solution was just alkaline. The globulin was then precipitated by the addition of an equal quantity of saturated ammonium sulphate solution, taken up in 0.6 per cent sodium chloride solution, and reprecipitated with ammonium sulphate. This process was repeated until seven precipitations in all had been made.

The fluid remaining after the first precipitation of globulin was filtered through a Seitz 'E-K' filter, and the perfectly clear filtrate, which would ordinarily contain the albumin fraction of the protein, was treated with 40 c c of 25 per cent sulphuric acid per litre, and allowed to stand overnight in the refrigerator. In no case did more than a trace of turbidity appear. Some of the protein solutions were saturated with ammonium sulphate, but no precipitate was obtained. It was concluded that the amount of albumin present in the vibrio proteins was very slight or nil.

The globulin, after the seventh precipitation, was taken up in distilled water, and dialysed first against running water for about ten days, and then against frequent changes of distilled water for three or four days until sulphates could no longer be detected. The dialysed material was filtered through a Buchner funnel to separate the pseudoglobulin and euglobulin fractions, and the latter was washed several times with distilled water. The filtrate, which contained the pseudoglobulin, was cooled in ice, and ice-cold alcohol added until a precipitate appeared. After standing for some hours in the refrigerator, the precipitate was filtered off, washed with alcohol and ether, and dried *in vacuo* over sulphuric acid. The euglobulin was suspended in water, and refiltered. The process was twice repeated, the euglobulin being taken up each time in a small quantity of water. After the second filtration the substance was again taken up in water, reprecipitated with alcohol, washed with alcohol and ether, and dried *in vacuo* over sulphuric acid.

PREPARATION OF THE SOLUTIONS

Exactly 0.5 g of the purified substance was taken in a 50 c c measuring flask, dissolved in 25 c c of normal NaOH (carbonate-free) and the solution made up to 50 c c, thus making a strength of N/2 of the alkali. A N/4 solution was also made by taking the same amount of the purified substance in 12.5 c c of the normal alkali, and making it up to 50 c c. Since the solutions showed a slight turbidity which would have interfered with the polarimetric readings they were filtered through Whatman's No. 5 filter-paper until clear. The final solutions were faintly yellow in colour, the colour faded out completely during the period when the

readings were being made. The solutions were kept at 37°C. The readings were made in a Schmidt and Hænsch polarimeter, using a 1 d tube, and sodium light. As indicated in Tables I to IV, the readings were made at frequent intervals during the first 48 hours, and at 24-hour intervals thereafter until the rotation had a constant value. This was reached after about nine days.

THE VIBRIOS USED

Proteins were prepared by the above method from the following six vibrios, two from each of the three vibrio groups we now recognize on the basis of their polysaccharide content and agglutination reaction (Linton and Shrivastava, 1934).

GROUP I Vibrios Nos 1612 and 1676. These organisms were derived from clinical cholera, and are morphologically typical vibrios. Number 1612 is a rough, non-agglutinating strain, and No. 1676 is agglutinable and smooth. The polysaccharide derived from them yields galactose as its characteristic sugar.

GROUP II Vibrios Nos 2027 and 505. These vibrios were likewise derived from clinical cholera, are morphologically typical vibrios and agglutinate with cholera anti-serum. The polysaccharide derived from them yields arabinose as its characteristic sugar.

GROUP III Vibrios Nos W880 and W3075. These vibrios were isolated from water in a cholera endemic area. They do not agglutinate with cholera anti-serum. Morphologically they are typical vibrios. Their polysaccharide yields arabinose on hydrolysis. Number W3075 is rough to Millon's reagent.

RESULTS

We may first consider the data which we have obtained from the vibrios derived from clinical cholera. The specific rotations are presented in Tables I and II, and in Graph 1, which is constructed from the data in them. From each of the four vibrios two fractions—pseudoglobulin and euglobulin—were derived, and the action of each in N/2 and N/4 alkali was studied.

A comparison of the eight sets of data presented in Table I shows that all of them correspond closely. The initial rotation appears to be about -80 , the rate of fall is the same, and the specific rotations finally reached are also identical. Only proteins which are identical would show these close similarities. All of the readings, for either concentration of alkali, can be satisfied by one curve. The pseudoglobulins and euglobulins in each protein seem identical. As would be expected, the more dilute alkali has a smaller effect upon the protein than the more concentrated, although the initial rotation appears to be about the same in both cases. The smaller effect is shown by the less rapid and less complete loss of rotatory power in the N/4 solution. Both the globulins from 1612 and 2027 show exactly the same degree of difference in the different solutions of alkali, i.e., the variation in concentration of alkali brings about an exactly equivalent variation in the rate at which the rotation is changed. In each case the readings were continued beyond the figures shown in the table to about 350 hours, but no further fall in the rotation was noted.

TABLE I

Specific rotations of the pseudoglobulin and euglobulin fractions of cholera vibrios Nos 1612 and 2027, in N/2 and N/4 NaOH

VIBRIO GROUP I (GALACTOSE CONTAINING) No 1612						VIBRIO GROUP II (ARABINOSE CONTAINING) No 2027					
Hours	Euglobulin		Hours	Pseudoglobulin		Hours	Euglobulin		Hours	Pseudo globulin	
	N/2	N/4		N/2	N/4		N/2	N/4		N/2	N/4
1	-78	-79				1	-77	-78			
						1			1	-76	-77
21	-72	-74	3	-70	-73	3	-71	-73	3	-70	-73
5	-65	-69				6	-64	-69	6	-64	-68
26	-43	-49	22	-45	-51	24	-44	-50	24	-44	-50
						30	-42	-48	30	-42	-48
48	-37	-43	46	-36	-43	50	-36	-42	50	-36	-41
96	-27	-33	95	-27	-35	94	-27	-34	94	-27	-34
			119	-25	-32	120	-25	-32	120	-25	-32
144	-24	-30	143	-23	-30	145	-23	-30	145	-23	-30
168	-21	-28	166	-21	-28	169	-21	-28	169	-21	-28
192	-20	-27	190	-20	-27	193	-20	-27	193	-20	-27
216	-20	-26	214	-19	-26	217	-19	-26	217	-19	-26
264	-19	-26	262	-19	-26	266	-19	-26	266	-19	-26

Table II contains the data for the other proteins derived from cholera vibrios

TABLE II

Specific rotations of the pseudoglobulin and euglobulin fractions of cholera vibrios Nos 1676 and 505, in N/2 and N/4 NaOH

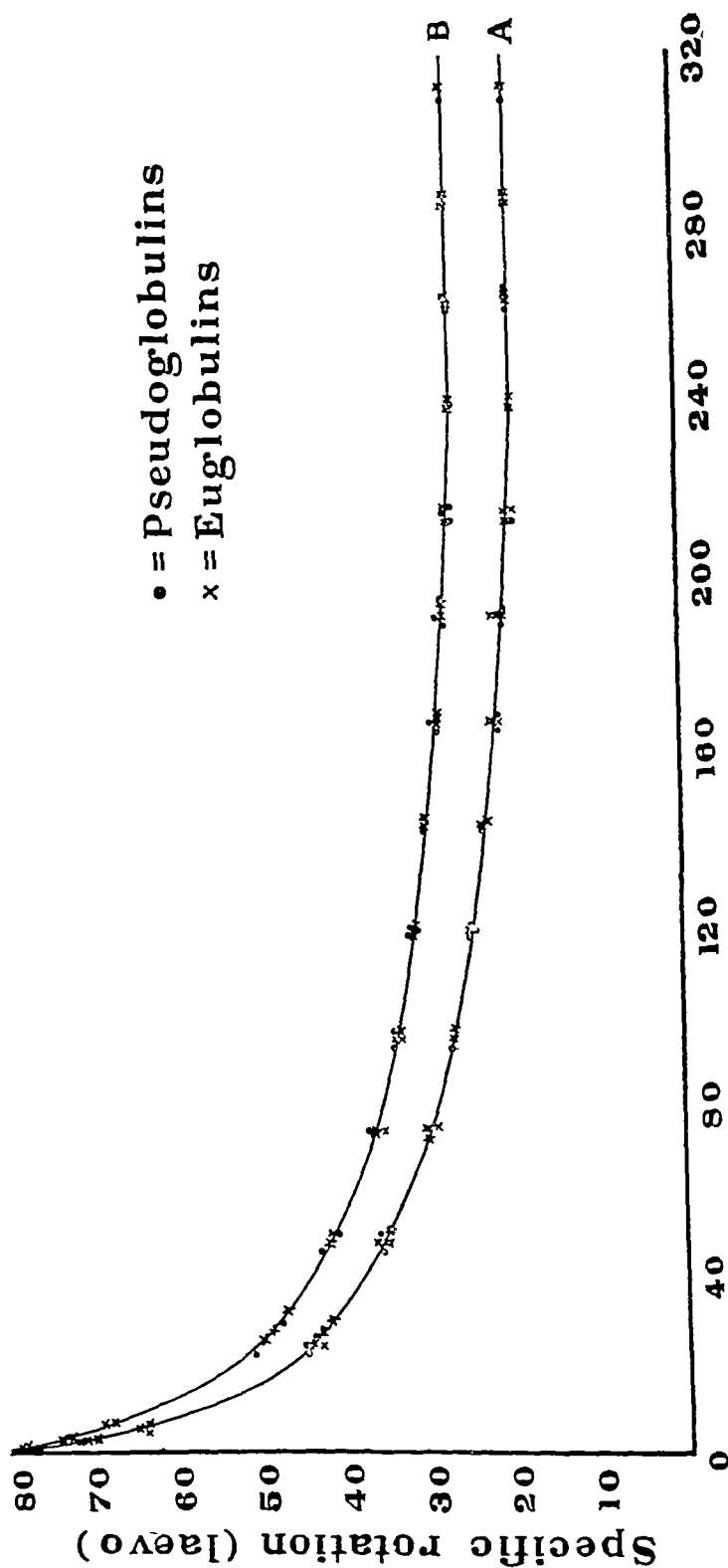
VIBRIO GROUP I (GALACTOSE CONTAINING) No 1676						VIBRIO GROUP II (ARABINOSE CONTAINING) No 505					
Hours	Euglobulin		Hours	Pseudoglobulin		Hours	Euglobulin		Hours	Pseudo globulin	
	N/2	N/4		N/2	N/4		N/2	N/4		N/2	N/4
									$\frac{1}{2}$	-77	-78
1	-77	-78	1	-77	-78	1	-76	-77			
2½	-71	-73	3	-70	-73	3	-70	-72	2½	-72	-74
5½	-64	-68	6	-64	-69						
24	-43	-50	26	-43	-50	24	-44	-50	24	-45	-50
48	-36	-43	50	-37	-44	48	-36	-42	48	-36	-42
72	-29	-35	72	-30	-37	72	-31	-36	72	-31	-36
			97	-27	-34	97	-27	-33	97	-27	-33
120	-25	-32	120	-24	-31	121	-25	-32	121	-25	-32
168	-22	-28	168	-22	-29	168	-21	-28	168	-21	-28
192	-21	-27	192	-20	-28						
217	-20	-26	217	-20	-26	215	-19	-26	215	-19	-26
240	-19	-26	240	-19	-26	240	-19	-26	240	-19	-26

Inspection of the figures in Table II shows that for either concentration of alkali a single curve can be drawn to satisfy the data both from the euglobulin and pseudoglobulin fractions of both proteins. These must then be identical. Further more, the curves given by the data in this table can be superimposed with complete concordance on the curves drawn from Table I. This has been done and the result is given in Graph 1.

We are able to conclude from this work that the proteins are identical in all these vibrios, and that in each of them the pseudoglobulins and euglobulins are also identical.

We may now turn to the data which have been obtained from the group III vibrios, i.e., vibrios derived from water, and possessing an arabinose-containing polysaccharide.

GRAPH 1



TIME IN HOURS

Curves representing the behaviour of the euglobulins (x) and the pseudoglobulins (•) of the proteins of cholera vibrios Nos 1012 and 1070 (galactose containing polysaccharide), and Nos 2027 and 505 (arabose containing polysaccharide) Curve A is given by the proteins in N/2 solutions of alkali, and curve B by the N/4 solutions In either concentration of all ali, the two globulins give similar readings for each of the four organisms The data are given in Tables I and II

TABLE III

Specific rotations of the pseudoglobulin and euglobulin fractions of water-vibrios Nos W880 and W3075, in N/2 and N/4 NaOH

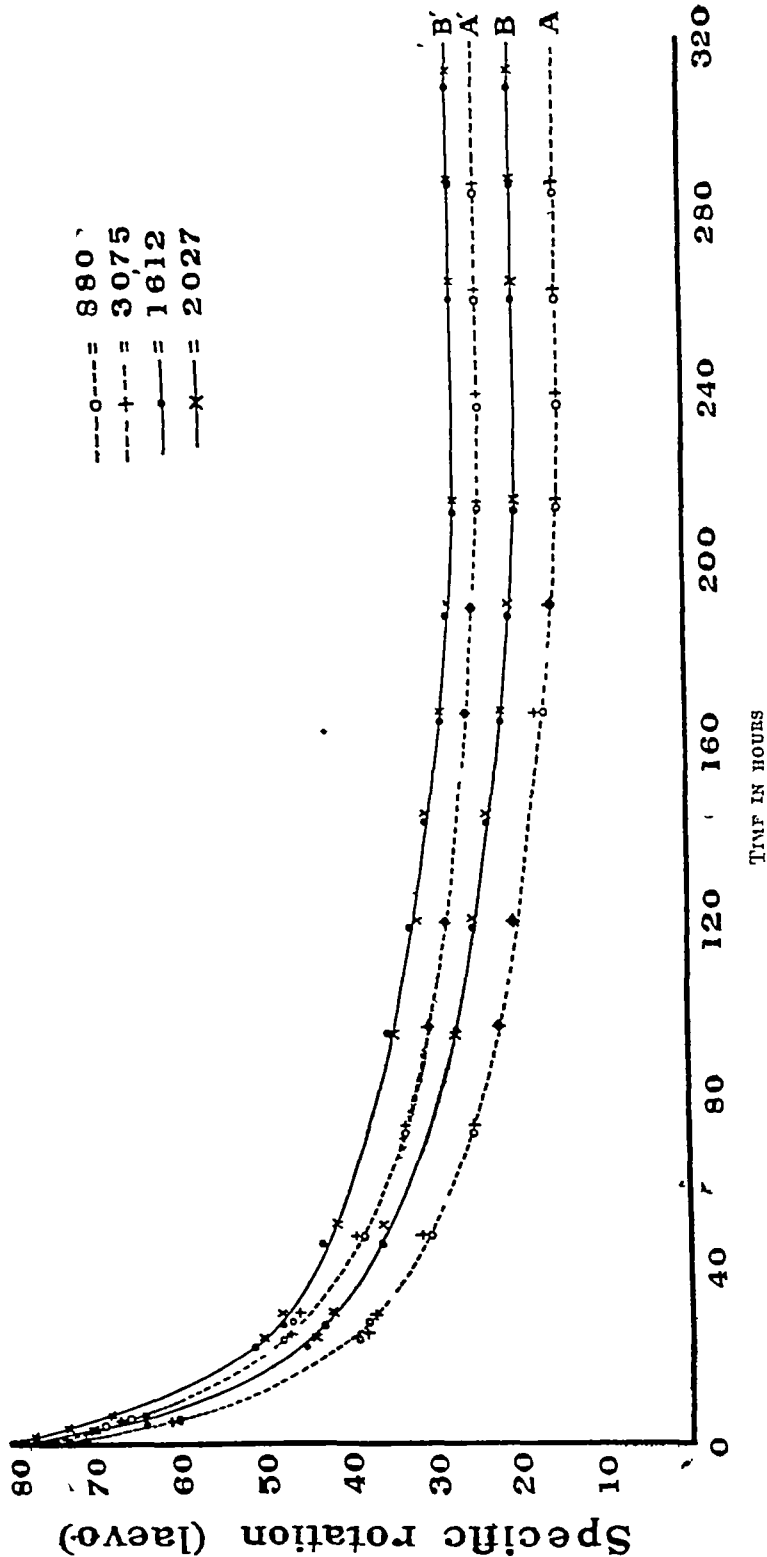
VIBRIO GROUP III (ARABINOSE CONTAINING)											
No W880						No W3075					
Hours	Euglobulin		Hours	Pseudoglobulin		Hours	Euglobulin		Hours	Pseudo globulin	
	N/2	N/4		N/2	N/4		N/2	N/4		N/2	N/4
$\frac{1}{2}$	-72	-74									
1 $\frac{1}{2}$	-70	-72	3 $\frac{1}{2}$	-64	-69	1 $\frac{1}{2}$	-70	-72	1	-71	-73
5	-60	-66	5	-60	-66	5	-60	-66	4 $\frac{1}{2}$	-61	-67
24	-39	-47	24	-39	-48	24	-39	-48	24	-39	-48
30	-37	-45	28	-38	-47	29	-37	-47	29	-37	-46
48	-31	-38	48	-30	-38	48	-31	-39	48	-31	-39
72	-24	-33	72	-25	-33	73	-25	-33	73	-25	-33
96	-21	-31	96	-22	-30	96	-22	-30	96	-22	-30
			120	-20	-28	120	-20	-28	120	-20	-28
168	-16	-25	168	-16	-25	168	-17	-25	168	-17	-25
191	-15	-24	191	-15	-24	192	-15	-24	192	-15	-24
215	-14	-23	214	-14	-23	216	-14	-23	216	-14	-23
263	-14	-23	262	-14	-23	264	-14	-23	264	-14	-23

As in the case of the other proteins, the readings were again continued to a period of about 350 hours, but no further change was noted. A study of the figures in Table III shows a complete concordance among the four sets of data given by the N/2 solutions. The N/4 solutions are also the same for both euglobulin and pseudoglobulin fractions of the two proteins. The four proteins from the water vibrios are then identical, and show an identical variation in behaviour in varying concentrations of alkali.

COMPARISON OF THE BEHAVIOUR OF THE PROTEINS IN ALKALINE SOLUTIONS

This comparison is made in Graph 2. The dotted lines represent the fall in rotation of the water-vibrio proteins in N/2 (curve A) and N/4 (curve A') alkali.

GRAPH 2.



TIME IN HOURS

Curves representing the behaviour of the pseudoglobulins of the water vibrio proteins (Nos W880 and W3075—arabinose containing) in comparison with the pseudoglobulins of the two groups of cholera vibrios. Curve A represents the pseudoglobulins of the water-vibrios in N/2 alkali, and curve A' the same fractions in N/4 alkali. It is evident that these two proteins are identical. The euglobulin fractions would give identical curves. Curve B' represents the pseudoglobulins of the cholera vibrios in N/2 alkali and curve B the same fractions in N/4 alkali. With different racemization curves the two groups of proteins cannot be identical.

The data for the cholera vibrio proteins in N/2 and N/4 alkali are given in curves B and B' respectively. It is evident that the two sets are different, and that the initial rotation, the rate of fall, and the level at which equilibrium is finally reached are distinct in the two groups of data. The curve for the water-vibrio proteins begins, for both concentrations of alkali, at about -75 , against -80 in the cholera vibrios, it falls more rapidly and reaches lower final levels of -14 against -19 in the N/2 solutions, and -23 against -26 in the N/4 solutions. It appears that the alkali acts more quickly and more nearly completely on the water-vibrio proteins than on the proteins of the cholera vibrios. The proteins therefore cannot be the same.

DISCUSSION

It is evident from the tables and graphs which have been given above that two distinct proteins are present in the vibrios which we have studied. The first of these, which we may designate as protein I, is found in both types of agglutinable vibrios from clinical cholera, i.e., the galactose- and arabinose-containing organisms. The second protein (protein II), which shows a more rapid loss^f of optical activity in alkaline solutions, is found in the water-vibrios.

We believe that we are now in a position to summarize our findings, in both the carbohydrate and protein analyses.

TABLE IV

A classification of the cholera and the cholera-like vibrios on the basis of the protein and carbohydrate analyses

GROUP I	GROUP II	GROUP III
Agglutinable vibrios from clinical cholera	Agglutinable vibrios from clinical cholera	Non agglutinable vibrios from water
Protein I	Protein I	Protein II
Polysaccharide I (galactose containing)	Polysaccharide II (arabinose containing)	Polysaccharide II (arabinose containing)

It should be emphasized that this table represents a summary of our researches, and is not to be taken as a final statement of a possible chemical classification. It would appear that the study on the chemical constitution is likely to advance considerably our knowledge of the vibrios and their relationships.

Groups I and II contain the same protein, and differ in their carbohydrate fractions. Groups II and III have the same polysaccharide fraction and different proteins. Groups I and III differ in respect to both constituents. From this point of view the vibrios of Group II are truly intermediate types: their polysaccharides are identical with those of the water-vibrios, and their proteins with those of the vibrios of Group I. Group I appears to contain the majority of the vibrios found in clinical cholera (Linton and Shrivastava, 1933c).

As Woodman (1921) has stated, 'the amount of difference between the optical values of two proteins in dilute alkali is not an absolute measure of the amount of structural difference existing between them, since the optical rotatory power of a complex substance is by no means a simple additive function of the activities of its several constituents'. Concretely, the differences in Graph 2 between the curves A and A' on the one hand and B and B' on the other do not indicate how nearly allied or how far apart the two proteins are in structure. All that can be concluded is that we are dealing with two structurally distinct proteins.

We have previously shown (Linton, Mitra and Shrivastava, 1934) that the nitrogen distribution was the same in the proteins which we now designate as proteins I and II. From this work we were led to conclude that the units from which the vibrio proteins were built up were identical. The present study shows that the arrangement of these units in the protein molecule is different, and that the proteins of Groups I and II on the one hand and of Group III on the other are distinct.

Two of the vibrios which we have worked with here have been rough to Millon's reagent, and have given smooth-rough colonies on agar (Nos 1612 and W3075).

The racemization method is now being applied to a further series of rough vibrios. The structure of the carbohydrates in the smooth and rough forms has previously been studied (Linton and Shrivastava, 1933*a* and 1933*b*), and the qualitative analyses given in those reports show that the carbohydrates in the two types of growth are the same.

Quantitatively, however, there is a difference between the smooth and rough forms. The transition to rough growth is accompanied by the loss of specific carbohydrate, and the degree of roughness appears to parallel this loss. In our earlier work we did not realize the extremely labile nature of smoothness in the vibrios, and their tendency to revert to a stage in which the amount of carbohydrate was only slightly, if at all, different from that of the corresponding rough forms. This tendency to reversion is especially marked under the conditions of mass growth which we use in our work. We therefore reported that differences did not exist between the amounts of carbohydrate in the smooth and rough forms, a statement which later work enables us to explain. Considerable differences do exist between the two forms of growth, when care is taken that smoothness is maintained. Under these conditions the smooth forms show generally from 6 per cent to 8 per cent of carbohydrate and the rough forms from 0.85 per cent to 2 per cent (dry weight). Grown in smaller quantities the differences might well be greater, due to the greater ease with which maximum smoothness or roughness could be maintained.

SUMMARY

The racemization method has been applied to the study of the proteins of the three groups of vibrios previously recognized on the basis of polysaccharide structure and agglutination reaction.

Two distinct proteins have been found in the three vibrio groups. Their distribution in relation to the polysaccharides may be summarized as follows —

Vibrio group I — The members of this group are composed of protein I and polysaccharide I. The group contains the majority of strains which we have found in clinical cholera.

Vibrio group II — The members are composed of protein I and polysaccharide II. These organisms also occur in clinical cholera.

Vibrio group III — These organisms are composed of protein II and polysaccharide II. They are water-vibrios.

The inter-relationships of these three groups, as far as they are now understood, are given in Table IV.

The euglobulin and pseudoglobulin fractions in any given vibrio protein are identical.

No albumin could be demonstrated in any of the vibrio proteins studied.

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Addendum.

The fourth possible combination of the two proteins and two carbohydrates which we have found in the vibrios has been disclosed through the analysis of an El Tor strain. This strain possesses the carbohydrate fraction of the Group I vibrios (Polysaccharide I), and the protein fraction of the Group III vibrios (Protein II). It therefore falls into a fourth group, and is related to the majority of vibrios found in clinical cholera through the possession of an identical carbohydrate, and to the water vibrios through the protein. Work now in progress on a series of El Tor vibrios will show whether this structural relationship holds throughout the group.

INVESTIGATIONS ON THE NUTRITIVE VALUES OF INDIAN FOOD-STUFFS

Part II

BY

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IN Part I of this series (Ghosh and Guha, 1933) we have reported the results of an investigation of the vitamins B₁ and B₂ and of the protein, iron, calcium and phosphorus contents of some Indian food-stuffs. The present paper records the results of further work in this line. The vitamin B₁-, B₂- and A-values of many Indian food-stuffs, including vegetables, pulses and fishes, have been investigated.

METHOD OF ASSAY OF VITAMINS B₁ AND B₂

The biological technique described before (Guha and Chakravorty, 1933, Ghosh and Guha, *loc cit*) has been used. The minimum quantity, which would produce a weekly gain in weight of approximately 10 g. for a period of 2 to 3 weeks in young vitamin B₂-deficient rats, has been taken to contain 1 unit of vitamin B₂ (Guha and Chakravorty, 1933). The vitamin B₁-values have been expressed in terms of international units by direct comparison with the international standard.

BIOLOGICAL TECHNIQUE FOR THE ASSAY OF VITAMIN A

Young rats, averaging 40 g. in weight, were kept in separate cages with screened bottoms and were fed on an artificial vitamin A-deficient diet, consisting of 65 per cent heated starch, 20 per cent 'light white' Casein (B. D. H.), 10 per cent lard, 5 per cent salt-mixture (McCollum) and 5 per cent dried yeast powder.

Each rat received in addition 2 drops of 'Rayneol' (irradiated ergosterol in vegetable oil of standardized potency) twice weekly.

An adequate supply of vitamins B₁, B₂ and D was thus ensured. According to the usual procedure, the vitamin A supplements were given when the growth-curve was either flat or falling on this dietary, showing a depletion of the vitamin.

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A reserves of the body As no international standard of vitamin A was available at the moment for comparison, one unit of vitamin A was defined to be the amount of food-stuff which would produce a weekly gain in weight of approximately 10 g for a period of 3 to 4 weeks under the above conditions The supplements were provided in dishes separately from the basal diet Each food-stuff was tested with two or more animals

RESULTS

The results are given in the following table —

TABLE

Serial No	FOOD STUFFS		Units of vitamin 'A' (in 100 g)	Units of vitamin 'B ₁ ' (in 100 g)	Units of vitamin 'B ₂ ' (in 100 g)
	Bengali names	Botanical names			
	VEGETABLES				
1	Alu (Namtal)	<i>Solanum tuberosum</i> (Legumi- nosæ)	{ 20 20 30	40	35
2	Am (Mango) (Bombai)	{ <i>Mangifera indica</i> (Anacardia ceæ)		14	33
3	„ (Langra)			16	35
4	„ (Fozli)		33	30	
5	Ash fruit	<i>Nephelium longana</i>		0	0
6	Begun (Egg fruit)	<i>Solanum melongena</i> (Solanaceæ)		20	100
7	Chal Kumrah	<i>Benincasa cerifera</i> (Cucurbita ceæ)		0	0
8	Hinche	<i>Enhydra fluctuans</i> (Compositæ)		28	10
9	Jamrool	<i>Eugenia malaccensis</i>		2	2
10	Kala Jam	<i>Eugenia jambolana</i> (Myrtaceæ)		0	0
11	Kanthai (Jack-fruit)	<i>Artocarpus integrifolia</i> (Arto carpaceæ)		20	16
12	Karolla	<i>Momordica charantia</i> (Cucur bitaceæ)		20	20
13	Khormuj (Phuti)	<i>Cucumis m'lo</i> (Cucurbitaceæ)		6	10
14	Kumrah	<i>Cucurbita maxima</i> (Cucurbi taceæ)		10	2
15	Kumrah Sak	Leaves of <i>Cucurbita maxima</i> (Cucurbitaceæ)		8	20
16	Lichoo (Indian Lichi)	<i>Nephelium lichi</i> (Sapindaceæ)		14	0
17	Mochā .	Spadix of <i>Musa paradisiaca</i> (Musaceæ)		2	0

TABLE—concl'd

Serial No	FOOD STUFFS		Units of vitamin 'A' (in 100 g)	Units of vitamin 'B ₁ ' (in 100 g)	Units of vitamin 'B ₂ ' (in 100 g)
	Bengali names	Botanical names			
	VEGETABLES—concl'd				
18	Pani phal	<i>Trapa bispinosa</i> (Roxburghii)		18	0
19	Polta	Leaves of <i>Trichosanthes dioica</i> (Cucurbitaceæ)		12	28
20	Ranga alu	<i>Ipomoea batatas</i> (Convolvulaceæ)		12	10
21	Shak alu	<i>Pachyrhizus angulatus</i> (Leguminosæ)		35	100
22	Ucheche	<i>Momordica charantia</i> (Cucurbitaceæ)		4	10
	CEREALS, PULSES, ETC				
23	Cholā dāl	<i>Cicer arietinum</i> (Leguminosæ)	66	15	66
24	Kancha mung (germ)	<i>Phaseolus mungo</i> (Leguminosæ)	30	10	16
25	„ (ungerm)		100	100	36
26	Masuri dāl	<i>Lens esculenta</i> (Leguminosæ)	45	40	25
27	Mator (Patnai)	<i>Pisum arvense</i> (Leguminosæ)	45	40	80
28	Sona mung	<i>Phaseolus radiatus</i> (Leguminosæ)	15	15	0
	FISH, ETC				
29	Bhetki fish	<i>Lates calcarifer</i>	33	16	4
30	Hilsa fish	<i>Clupea ilisha</i>	4	10	8
31	Katla fish	<i>Catla catla</i>	20	0	27
32	Kholsa fish	<i>Trichogaster fasciatus</i>	33	33	25
33	Koi fish	<i>Anabas testudineus</i>	20	10	2
34	Magur fish	<i>Clarias batrachus</i>	10	8	33
35	Mrigel fish	<i>Cirrhina mrigala</i>	33	10	0
36	Parse fish	<i>Mugil parsia</i>	60	10	16
37	Rohit fish	<i>Labeo rohita</i>	83	18	50
38	Shing fish	<i>Saccobranchius fossilis</i>	25	0	20
39	Shole fish	<i>Ophicephalus striatus</i>	20	0	2
40	Tangra fish	<i>Aoria tengara</i>	77	0	2

SUMMARY.

Of the forty Bengali food-stuffs investigated, Kancha-mung (*Phaseolus mungo*), Masuri-dal (*Lens esculenta*), Mator-dal (*Pisum arvense*) are found to be the richest sources of vitamin B₁. Begun (Egg fruit), Shak-alu (*Pachyrhizus angulatus*), Chola-dal (*Cicer arietinum*) and Mator-dal are fairly good sources of vitamin B₂. Among the fish-bodies investigated, those of Rohit (*Labeo rohita*), Parse (*Mugil parsia*) and Tangra (*Aoria tergara*) were found to be rich in vitamin A. It should, however, be pointed out that these fishes cannot compare with fish-liver oils in their potency for vitamin A.

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OBSERVATION ON THE CHEMISTRY OF THE OXYTOCIC HORMONE OF THE PITUITARY GLAND

Part II

BY

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IN an earlier communication (Guha and Chakravorty, 1933) the behaviour of preparations of the oxytocic hormone of the posterior lobe of the pituitary gland to various chemical treatments has been described. Further work is reported in this paper. Preliminary results obtained with phosphotungstate fractionation at different hydrogen-ion concentrations and with silver nitrate-baryta fractionation indicate possibilities of concentrating the active principle by such methods. Experiments on adsorption by different preparations of alumina and by barium sulphate are also described.

EXPERIMENTAL

The present work was carried on with a sample of dry powdered extract of posterior lobes of bovine pituitary glands prepared according to the method previously described (Guha and Chakravorty, *loc cit*).

The oxytocic activity of each of the preparations was tested on the isolated uterus of the virgin guinea-pig according to the method given by Burn and Dale (1922) with slight modifications (Guha and Chakravorty, *loc cit*). Ringer's solution prepared from glass distilled water was always used, and all reagents employed were of Merck's extra pure quality.

The greatest difficulty was faced in selecting suitable uterine muscles. In our experiments guinea-pigs weighing 300 g to 350 g were found most suitable. The standard doses, with which comparisons have been made, have always been chosen so as to produce sub-maximal contraction of the uterus. Standard doses have

lamp (Hanovia Home Sun, working at 220 volts and 2 amperes) at a distance of 15 cm for 1 hour

The extract gradually became opalescent and finally became clear and granules of solid matter separated out. The precipitate was centrifuged off and the clear centrifugate made up to 10 c c [P (72)]

Inactivation with 2N NaOH at room temperature—In order to find out if our pituitary extracts were contaminated with histamine the following experiment was carried out —

Five c c of the original pituitary extract P (84) were treated with an equal volume of 2N NaOH and allowed to stand at room temperature for 2 hours. The sodium hydroxide was then neutralized by adding concentrated HCl. The volume was made up to 15 c c [P (103)]

The results of the tests are summarized in the following table —

TABLE.

Number of preparations	Description of preparations	Percentage of activity retained in the fractions tested
P (67)	Phosphotungstate fraction at pH 5.0	85
P (68)	" " " pH 3.0	0-10
P (69)	" " " pH 1.0	0
P (70)	Final filtrate from phosphotungstate fraction at pH 1.0	0-10
P (61)	Silver baryta fraction at pH 6.5	80
P (62)	Filtrate from silver baryta fraction at pH 6.5	10
P (100)	Filtrate after adsorption with freshly precipitated Al_2O_3 (hydrated)	20
P (73)	Filtrate after adsorption with Al_2O_3 (Merck, hydrated, free from alkali)	90
P (74)	Al_2O_3 eluted with H_2CO_3	0
P (82)	Filtrate from BaSO_4 adsorption	50
P (72)	Irradiation with ultra violet rays	0
P (103)	Treated with 2N NaOH for 2 hours	0

SUMMARY

(1) Considerable concentration of the oxytocic principle appears to be effected by phosphotungstate fractionation at pH 5.0 and silver nitrate-baryta fractionation at pH 6.5

(2) The hormone was fairly well adsorbed by freshly precipitated alumina but not by Merck's alumina (hydrated, free from alkali). Barium sulphate also adsorbed the hormone to some extent

(3) The hormone was inactivated by ultra-violet irradiation

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CULTIVATION OF THE GONOCOCCUS FOR VACCINE

BY

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THE gonococcus is usually considered to be a more delicate organism than most pathogenic cocci, and to have special requirements in regard to its conditions of growth and preservation on artificial media. Many workers have devised special media for its cultivation and have investigated the physical conditions necessary for growth and the nutrient constituents required.

The media described have been often of a complicated nature and have contained ascitic fluid, serum, whole blood (human and animal), testicular substance, etc., and stress has been laid on the importance of reaction, temperature of incubation, and the physical state of the medium. Our own experience with this organism, in connection with its cultivation and the preservation of strains for vaccine purposes has indicated that comparatively simple media can be employed provided that certain physical conditions of growth are attended to.

The following notes are intended to illustrate practical experience in working with this organism —

PHYSICAL CONDITIONS REQUIRED

(a) *Temperature of incubation* — Jenkins (1921) gives the optimum temperature for growth as somewhat below 37.5°C, the most luxuriant growth being obtained at 35°C to 36°C.

Our stock organisms are grown at 37°C, the growth being satisfactory and luxuriant at this temperature.

(b) *Moisture* — A moist medium and usually one containing water of condensation has been considered necessary by most workers especially in regard to obtaining primary cultures. For the maintenance of a moist surface, Cook and Stafford (1921) incubated their cultures in a glass-jar containing a beaker of water. For the same purpose Tulloch (1929) used a solution of brilliant green.

Certain of our strains which, when freshly isolated, were difficult to grow developed satisfactorily when incubated in a sealed glass-jar in which a filter-paper soaked with 1 in 500 solution of perchloride of mercury was placed.

There was a tendency to the development of moulds on account of the excessive moisture, but this was easily eliminated by pushing down the cotton-wool plug and placing a pledget of wool moistened with the perchloride lotion over it

(c) *Medium* —A blood agar prepared in the following way was used for stock cultures

Ten c c of Douglas broth agar (trypsin digest of mutton with 3 per cent agar) with pH 7.6 is placed in each test-tube and sterilized at 120°C for one hour. The tubes are cooled to 45°C and 1 c c of pigeon's blood added to each. The blood is rapidly mixed by rotating the tubes which are then sloped. The pigeon's blood provides an abundant supply of peroxidase and is especially favourable to growth of the gonococcus.

The growth and the viability of six strains of the gonococcus isolated locally were tested on the above medium in comparison with five other media. The results are shown in Tables I and II.

It will be seen that growth on the Douglas broth agar with pigeon's blood was satisfactory although not quite so heavy in 24 hours as on chocolate blood agar. Survival at 37°C was on the whole longer than on the other media tested, the average period being 20 days.

To determine the comparative yield for vaccine purposes the following tests were carried out —

Twenty-five c c each of pigeon blood agar, serum agar, papain digest blood agar, and chocolate blood agar were placed in Petri dishes giving a surface area of 38.5 square centimetres. Each plate was inoculated with 1,200 million gonococci from a 24-hour culture suspended in 0.4 c c of normal saline. The yield was estimated after 24 hours' incubation at 37°C.

The respective yields were —

Medium	Mg dried bacterial substance per sq cm
1 Pigeon's blood agar	0.24
2 Douglas agar and normal horse serum	0.16
3 Papain's digest agar and pigeon's blood	0.18
4. Chocolate blood agar	0.18

The high yield on pigeon's blood agar shows the suitability of this medium for use in preparation of vaccine material.

TABLE I.

12 stock strains of gonococcus after 24 hours' incubation at 37°C

	A 47-2	A 47-3	A 47-4	A 47-5	A 47-6
	Small, convex transparent greyish colonies about 1 mm in diameter	Small pin point colonies almost making a homogeneous film	Small pin point colonies making a homogeneous film	Homogeneous film of confluent colonies making a thick film	Small discrete circular convex colonies about 1 mm in diameter
	Circular raised, convex colonies about 2 mm in diameter showing a luxuriant growth	ditto Growth a little more luxuriant	ditto	Circular raised transparent colonies about 1½ mm in diameter	ditto
2 Papain digest agar + 10 per cent pigeon's blood	* No growth after 24 hours, a thin homogeneous film after 48 hours				
3 Douglas agar + 10 per cent normal horse serum	* No growth in 24 hours, a thin homogeneous film after 48 hours	Luxuriant growth, a homogeneous film of confluent colonies	Small pin point discrete circular convex colonies showing typical umbo in the centre	Small discrete transparent raised convex colonies about 1 mm in diameter	ditto
4 Douglas agar + 1 per cent yeast extract + 0.1 per cent haematin	* No growth in 24 hours, a thin film of colonies in the upper third of medium after 48 hours, growth very poor	Small pin point circular raised transparent colonies	A few large size colonies about 2 mm in diameter	ditto	ditto
5 Chocolate blood agar	A homogeneous film showing luxuriant growth	Continuous film of colonies, good growth	A homogeneous film	A thin homogeneous film	A thin homogeneous film
6 Fildes agar	A homogeneous film	A few large size colonies about 2 mm in diameter with irregular margins	ditto	ditto	ditto

* These two were delicate strains which had presented difficulty in primary culture and for satisfactory subculture the jar method was required

TABLE II

*Viability of the six stock strains of gonococcus on different media
in days at 37°C*

Media	STRAINS OF GONOCOCCI					
	A 47-1	A 47-2	A 47-3	A 47-4	A 47-5	A 47-6
1 Douglas agar + 10 per cent pigeon's blood	21	23	21	11	23	23
2 Papain digest agar + 10 per cent pigeon's blood	23	19	23	6	21	26
3 Douglas agar + 10 per cent normal horse serum	23	25	17	23	17	9
4 Douglas agar + 1 per cent yeast extract + 0.1 per cent hematin	6	10	23	23	17	11
5 Chocolate blood agar	17	17	26	26	12	11
6 Filde's agar	10	14	8	8	8	26

MAINTENANCE OF STRAINS

For keeping gonococcus strains for prolonged periods without subculture Torrey and Buckell (1922) described a semi-solid medium which contained accessory growth principles. This medium is somewhat elaborate to prepare and a trial of a semi-solid medium of a simpler type based on a modification of the pigeon's blood agar described has shown that this will be satisfactory for the purpose.

The semi-solid medium is prepared as follows —

Eighteen c.c. of 3 per cent Douglas agar is melted and added to 450 c.c. of sterile Douglas broth. This is poured into sterile test-tubes in 9 c.c. quantities and again sterilized at 120°C. The tubes are cooled to 45°C and 1 c.c. of pigeon's blood added to each.

The tubes are inoculated at the upper level of the medium and sealed with paraffin wax over the plug.

A second medium was also prepared consisting of 15 per cent gelatine in Douglas broth with the addition of 1 c.c. of pigeon's blood to each 9 c.c. of medium.

The survival period of five strains of the gonococcus in these two media after incubation continuously at 37°C is shown in Table III

TABLE III

Viability of the five stock strains of gonococcus on the two semi-solid media in days at 37°C.

Media	STRAINS OF GONOCOCCUS				
	A 47-1	A 47-2	A 47-3	A 47-4	A 47-5
1 Semi solid pigeon's blood agar	92	75	75	84	82
2 Pigeon's blood gelatin	75	63	62	75	53

The semi-solid pigeon's blood agar gave longest survival, the period varying from two and a half to three months

SUMMARY

1 Simple media for the cultivation and maintenance of strains of the gonococcus for vaccine purposes are described

2 A pigeon's blood agar has been found suitable for obtaining primary isolations and to give a satisfactory yield for vaccine

3 A semi-solid modification of this medium maintains strains for two and a half to three months without subculture

4 The importance of a sufficient supply of moisture in the case of delicate strains is noted and a method of ensuring this is detailed

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These were difficulties which there was no way of surmounting with the facilities available to us

As it was not known in what form blood was likely most efficiently to conserve the infective principle it was decided to send it in the three following forms —

- (a) Citrated whole blood, i e , 5 c c of blood in 15 c c of 2 per cent sodium citrate solution
- (b) Glycerinated whole blood, i e , 5 c c of blood in 5 c c of 50 per cent glycerine
- (c) Citrated-glycerinated blood, i e , 5 c c of (a) mixture in 5 c c of 50 per cent glycerine

As the work was carried on during the middle of the hot weather, the journey from Peshawar to Kasauli occupied approximately 24 hours, and the blood was taken on the day previous to its despatch, and was sent either by messenger or post without any special precautions for cooling it, it will be evident that there was every likelihood of any but a fairly resistant virus dying out between the taking of the blood at Peshawar and its use in Kasauli. The average interval elapsing between the two operations was sixty hours

PREVIOUSLY RECORDED LABORATORY FINDINGS

The most important laboratory findings as recorded by previous workers on sandfly fever are enumerated below and will later be considered in more detail in relation to our own observations

- 1 *Blood taken from a patient on the first day of the fever when inoculated into man produces a typical attack of the fever after the usual incubation period* (Doerr, Franz and Taussig, 1909, Birt, 1910)
- 2 *Blood passed through a fine filter can still convey the typical fever when inoculated into a susceptible person* (Doerr, Franz and Taussig, 1909, Birt, 1910)
- 3 *Phlebotomus flies fed upon a patient suffering from the fever, when conveyed to a non-endemic area and fed on susceptible persons, convey the disease to the latter* (Doerr, Franz and Taussig, 1909, Birt, 1910)
- 4 *The flies do not become infective until seven to ten days after their meal on a fever case* (Doerr, Franz and Taussig, 1909, Birt, 1910)
- 5 *The incubation period varies between three and a half days and seven days* (Doerr and Russ, 1909, Birt, 1910)
- 6 *The virus retains its infectivity for a week in vitro* (Birt, 1910)
- 7 *Experimental animals are immune to the disease* (Birt, 1910)
- 8 *No visible causative agent has been demonstrated in the blood by direct microscopical or cultural examination* (Doerr, Franz and Taussig, 1909, Birt, 1910)
- 9 *A Leptospira has been isolated from cases of so-called sandfly fever* (Whittingham, 1921, Vervoort, 1922)

In order to give the clearest account of our own findings and to indicate where they confirm or differ from those recorded by the other workers referred to it has

seemed to us most convenient to deal with them *seriatim* under the headings we have used above in summarizing the conclusions of these workers

1 *Blood taken from a patient in the first day of the fever when inoculated into man produces a typical attack of the fever after the usual incubation period*

The impression one gathers from the literature quoted is that the virus has disappeared from the peripheral circulation by the second day of the disease. It was on this account that the majority of the blood samples sent to Kasauli from Peshawar for test were taken on the first day of the disease. In Table I given below are recorded the results of inoculating the bloods into volunteers in Kasauli from the point of view of the infectivity of the blood on the first and second days of the fever —

TABLE I

Showing the number of infections obtained by inoculating into volunteers the blood of sandfly fever cases taken on the first and second days of the attacks

	Number of bloods used	Number of cases of fever produced	Typical	Modified *
First day bloods	10	7	6	1
Second day bloods	4	2	1	1
TOTALS	14	9	7	2

* *Modified cases* — No case was considered as a 'positive' unless there was actual fever. Those cases in which the fever was only of moderate intensity and short duration were called 'modified cases'. The discomfort, in the form of various aches, pains and general malaise of some of the cases was considerable and often lasted for some days beyond the actual duration of the fever.

It will be seen from the table that, contrary to the impression conveyed by previous work, there is probably a considerable percentage of cases in which blood is still infective on the second day of the fever. No investigation on this point was made beyond the second day of fever.

The dose of the inoculum varied with the concentration of the blood, an attempt being made in each case to give the equivalent of 0.7 c.c. of whole undiluted blood.

As regards the best method of sending blood for the experiments the numbers used were too small for us to come to any decision but, in practice, successful transmissions of fever to man or monkey were produced by all the methods mentioned as well as by direct inoculation of fresh whole blood from fever cases to volunteers. The latter process was carried out by inoculating the blood of fever cases produced in Kasauli from the Peshawar bloods into a second series of volunteers. The actual figures for these experiments are given below in Table II —

TABLE II

Showing the number of infections of sandfly fever produced in man by blood inoculation according to the form in which the blood was used

Condition of blood	Number of bloods inoculated	Number of fever cases produced	Typical	Modified
Citrated whole blood	14	7	5	2
Glycerinated whole blood*				
Citrated glycerinated blood	10	2	2	
Fresh whole blood	1			
TOTALS	25	9	7	2

* Glycerinated whole blood was used for infection in monkeys only

2 *Blood passed through a fine filter can still convey the typical fever when inoculated into a susceptible person*

The quantities of blood received by us were comparatively small and, being mostly used in the direct inoculation experiments, not much was available for filtration. Such experiments as were done, however, showed that the infective principle was capable of passing a comparatively fine filter and retaining its virulence. The filters used by us were L3 and L5 Chamberland candles.

The results of the experiments are given in Table III. The dose of filtrate used for inoculation varied from 3.8 c.c. to 10 c.c. —

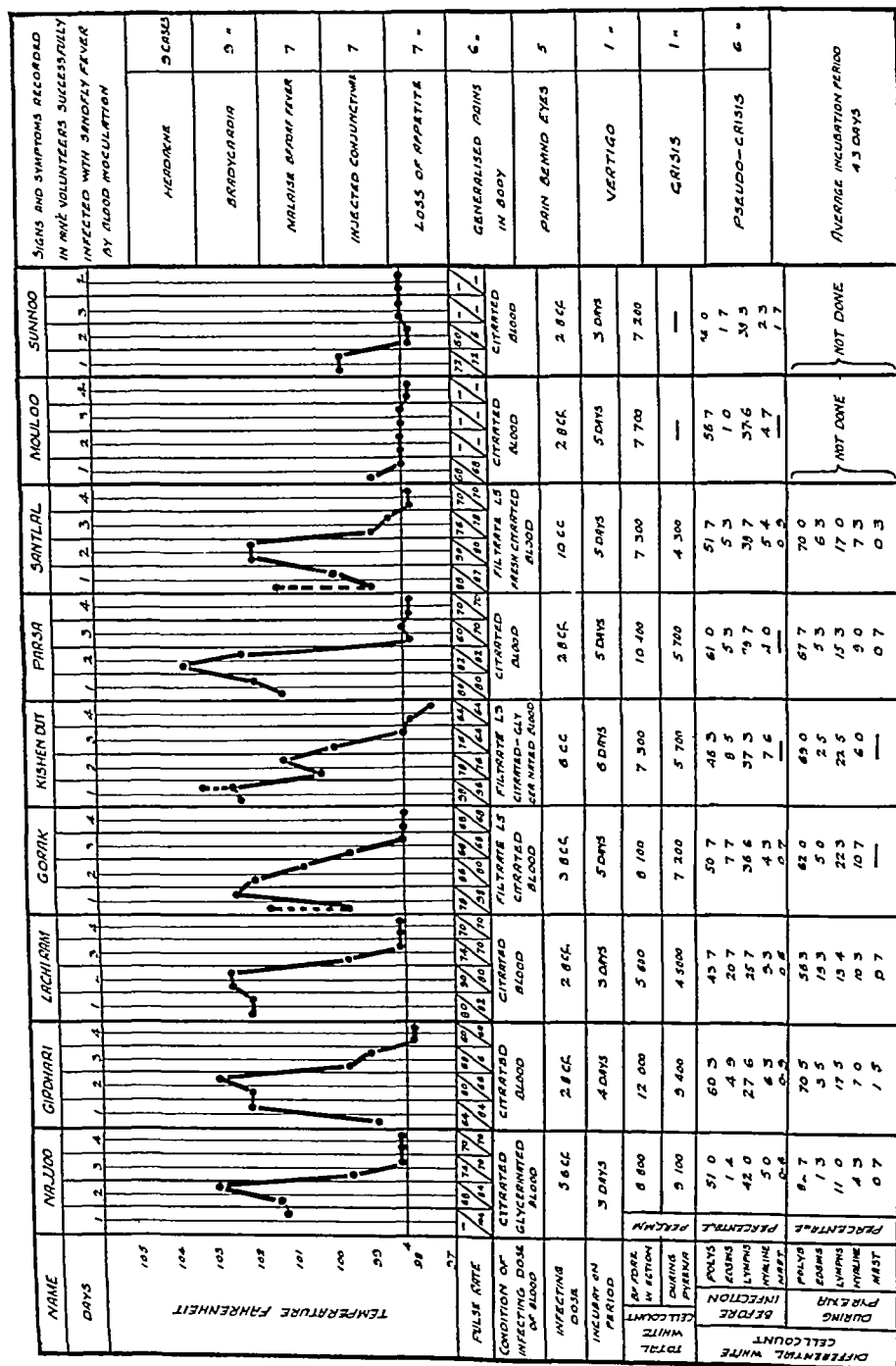
TABLE III

Showing the results of filtration experiments

Condition of blood before filtration	Number of filtrates inoculated	Number of fever cases produced	Typical	Modified
Citrated whole blood	4	2	2	
Citrated glycerinated blood	2	1	1	
TOTALS	6	3	3	

The temperature charts of all the cases of sandfly fever induced in Kasauli by the inoculation of blood, whatever its form, or filtrates of blood, are shown in Chart 1 —

CHART I

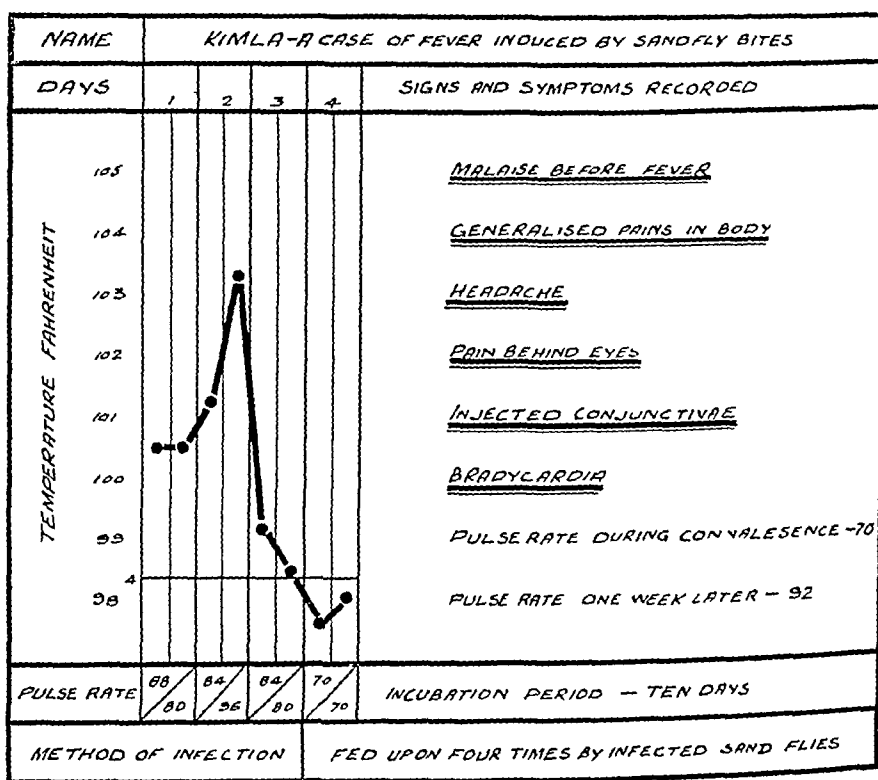


The temperature charts of, and other clinical observations made on, cases of sandfly fever induced in volunteers by inoculation of infective blood

3. *Phlebotomus* flies fed upon a patient suffering from the fever when conveyed to a non-endemic area and fed on susceptible persons convey the disease to the latter

Eight batches of laboratory-bred *P. papatasi* which had been fed on cases of sandfly fever at Peshawar or Landikotal were received by post or passenger train in Kasauli. These flies were sent in the mosquito despatch boxes devised by Barraud (1929). The total number of flies despatched was 110. Of these 69 arrived alive, 32 dead and 9 were not accounted for. Of the 69 flies which arrived alive only 18 fed on volunteers. Eight volunteers were used. As the flies when received in Kasauli had fed only three or four days previously it was considered that the third and subsequent feeds were most likely to be the infective ones. The second feed (or first in Kasauli) was given to keep the flies alive and the third and subsequent

CHART 2



Sandfly fever induced by bites of sandflies

feeds were on the most suitable volunteers. Of the eight volunteers all were fed upon once, five were fed upon twice, one was fed upon three times, one was fed upon four times and one was fed upon five times.

Two volunteers developed fever, their histories being as follows—

Volunteer Ram Das, age 9, male—On 22nd August three flies which had originally fed on sandfly fever cases in Landikotal between the 13th and

16th August were fed upon the volunteer. One of the flies took only a partial feed. This fly was again fed on the volunteer on the succeeding day.

On 25th August the volunteer developed fever which lasted for three days. Unfortunately, the boy lived in a village and did not report sick until the third day so that no record of his temperature was made. When seen on the 27th August the fever was subsiding and the temperature was normal on the morning of 28th August. On the 27th the patient's face was flushed, the eyes were suffused and he complained of pains all over the body and headache. The pulse rate was slow, 80 per minute. No coryza, cough or any other symptoms which could account for the fever were present at any time. We have no doubt in our minds that this was a genuine case of sandfly fever transmitted by the bites of sandflies fed at Landikotal six to nine days previously on sandfly fever cases. It is unfortunate that owing to the non-reporting of the illness the temperature was not recorded.

Volunteer Kımila, age 11, male—On 21st September one fly which had originally fed on a sandfly fever case in Landikotal on 16th September was fed upon the volunteer. The feed was partial. On 22nd, 23rd and 24th September three, two and two flies respectively of the same batch were fed, all these feeds were partial.

On 5th October, 10 days after the last feed, the volunteer complained of fever. The fever lasted for three days. There was complaint of pain behind the eyes, which were strongly injected, pains all over the body and headache. The pulse rate was markedly slow considering the degree of fever and the age of the patient, being 80 per minute. Chart 2 shows the progress of the fever in this case.

If this case was a genuine one of sandfly fever the incubation period was longer than usual and for this reason we make the diagnosis with reserve.

4 *The flies do not become infective until seven to ten days after their meal on a fever case*

In the two cases of fever recorded by us above the flies may be said to have become infective between the minimum and maximum of six days and eight days.

5 *The incubation period varies between three and a half and seven days*

In the case of fevers following feeds by infected sandflies the incubation period varied in the two cases recorded by us between minima and maxima of three days and 10 days. In our series of inoculation experiments the minimum and maximum incubation periods for typical cases of the fever, as measured by the interval elapsing between the time of inoculation and the first onset of fever, were three and six days, the average time for the seven positive typical cases being 4.4 days. In one of the two modified attacks the incubation period was three days and in the other five days.

6 *The virus retains its infectivity for a week in vitro*

In all our experiments we endeavoured to use the bloods sent from Peshawar as soon as possible after receipt as we were not, at the time, especially concerned to see how long the virus survived *in vitro*, but rather to utilize the blood while the virus was still alive

In one case, however, 10 c c of the filtrate from a L13 Chamberland candle from a mixture of specimens of citiated whole blood and citrated-glycerinated blood of a sandfly fever case, kept in the ice-chest for 14 days, was inoculated into a monkey and appeared to be virulent. This experiment is dealt with below in the section dealing with animal experiments

7 *Experimental animals are immune to the disease*

We have no conclusive evidence to offer either for or against this statement but certain of our experiments would appear to suggest that at least in the case of monkeys it may be possible to reproduce the disease by inoculation

The experiments conducted by us consisted in passing human blood of sandfly fever cases into monkeys (*Macacus rhesus*), and monkey blood into humans and monkeys. For convenience these different experiments will be considered *seriatim* in the groups indicated above but, before doing so, it will be necessary to make a few remarks on certain fallacies which had to be guarded against and various factors which had to be taken into consideration in interpreting our results

In a disease such as sandfly fever, where one is dependent for diagnosis on clinical signs and subjective symptoms, it is essential that these signs and symptoms should be typical and unequivocal before the assertion is made in any particular case that the condition induced as the result of a certain experiment is really sandfly fever. When one is dealing with animals the subjective symptoms are ruled out as unascertainable and diagnosis has to be made on clinical signs alone. This would be fairly satisfactory were they present in typical form and degree but, if we are dealing with an animal but mildly susceptible to the disease, it is likely that the signs as well as the symptoms will be modified in kind or degree or both. This is the less remarkable when we consider that in man himself, who is highly susceptible to sandfly fever, modified attacks may and do occur. It is on account of these considerations that we lay no claim actually to have transmitted the disease to monkeys but merely point out that some of our experiments suggest that such was the case, albeit the disease was mild in degree and the signs more evanescent than in the more highly susceptible human being

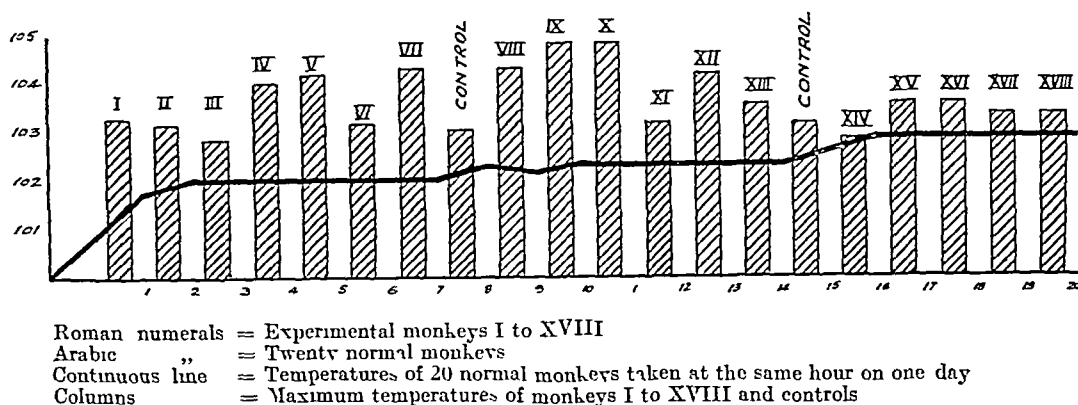
The main factors on which we had to rely in making our diagnosis in the case of monkeys were the temperature and the incubation period and the latter had to be gauged by the former alone

Two of us have had a very large experience of dealing with monkeys owing to the numbers used by us in experiments in connection with rabies and we were therefore fully cognizant of the variations in temperature exhibited by monkeys and it became essential to include an elaborate system of controls in our experiments. To do this we decided to take all the temperatures at the same hour each day, to take the temperatures of normal monkeys at the same time, and to employ control

monkeys into which normal monkey and human bloods had been inoculated in the same amounts as the infected blood into the experimental monkeys. In addition, the temperatures of 20 normal monkeys were taken at the same time in order to establish a provisional mean normal temperature for the time of day and other conditions prevailing.

The continuous **bold** line in Chart 3 shows graphically the temperatures of 20 normal monkeys taken at the same time of day (4 p.m.) superimposed on the maximum temperatures of 18 of the experimental monkeys used in this investigation. It will be seen that the variations lie within one degree Fahrenheit. The average temperature of these 20 monkeys taken at the time stated was 102.3°F.

CHART 3



Experiments in which human blood of sandfly fever cases was inoculated into monkeys—The human blood specimens used in these experiments were in some cases sent from Landikotal or Peshawar and had therefore undergone a three-day exposure to the heat of the plains as well as a long railway journey. In other cases the specimens were freshly drawn blood from cases of sandfly fever induced in Kasauli by inoculation of the Peshawar bloods.

The only clinical indication available of successful infection of the monkeys was a rise of temperature which persisted for some time above the point considered the daily maximum under normal conditions. As a control against the possible thermogenic effect of inoculation of blood alone certain controls were given inoculations of normal human blood. This was found actually to cause a slight rise of temperature and therefore cases in which supposedly infective bloods caused no greater rise than in these controls were not looked upon as positive results. This, however, does not rule out the possibility that their blood may have contained the virus, as certain of our results seemed to indicate this as a possibility. Thus, the blood of monkeys I and II in which the temperature rose only slightly above 103°F (although this was higher than the normal human blood control monkeys) produced definite fever in monkeys IV, V and VII and therefore presumably contained the virus.

Using, then, temperature as our criterion we found that out of 11 monkeys inoculated with the blood of sandfly fever cases five contracted definite fever, while six showed no fever or only a transient rise as compared with the control monkeys which received an inoculation of normal human blood

In combined Chart 4 we have given the temperatures of these five monkeys which appeared to show a definite short fever of greater degree than would be caused by injection of normal human blood alone. The temperature chart of a monkey receiving an equal amount of normal human blood is also given for comparison, and, as a background to the charts, the provisional normal temperature for monkeys is given as a straight line at 102.3°F

A glance at the individual charts will show that there is a distinct difference in temperature between them and the control monkey in the case of monkeys VIII, IX, and X but much less in the case of monkeys I and II

The incubation period appears shorter than in the case of similar experiments with human beings but it has to be remembered that a much greater volume of blood relative to body weight was inoculated in the case of the monkeys

Experiments in which supposedly infective monkey's blood was inoculated into monkeys Seven experiments were carried out in this series. In each case the monkey received into the loose tissues of the abdomen 5 c c of freshly drawn blood of a monkey infected from a human case. Three out of the seven monkeys became infected judging by the criteria laid down by us for the animal experiments

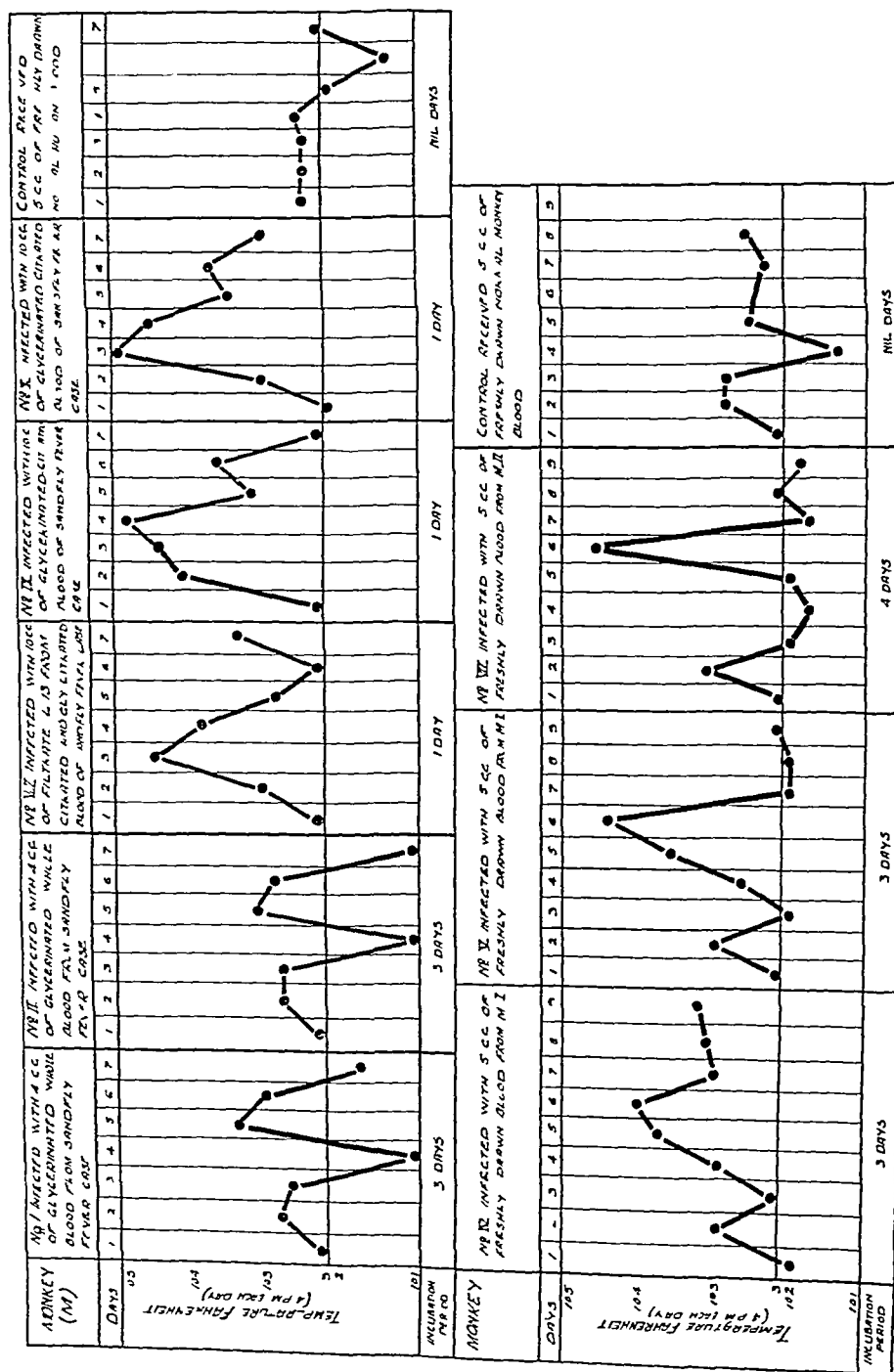
The charts of these monkeys are given in combined Chart 4 together with the chart of one of the control monkeys inoculated at the same time with the same amount of normal monkey blood

It will be seen that there is an incubation period of three or four days and that the fever lasted for one to five days. The control monkey showed the relatively small rise of temperature which has been previously mentioned

Experiments in which supposedly infective monkey's blood was inoculated into humans—Three such experiments were carried out. In each case the monkey used had been infected by means of blood sent from Peshawar. In two of the cases 10 c c of citrated-glycerinated blood, sent as such from Peshawar, was used for infecting the monkeys (monkeys IX and X). In the third case the infection of the monkey (monkey VIII) was brought about by 10 c c of a filtrate through an L13 Chamberland candle of a mixture of citrated blood and citrated-glycerinated blood of the same individual. The filtration was performed in Kasauli after receipt of the bloods from Peshawar. All these monkeys became infected, as judged by the criteria for infection in animals we have already discussed. Their temperature charts are shown on combined Chart 4. From each of these monkeys 5 c c of freshly drawn whole blood was inoculated into a human volunteer in the loose tissues of the abdomen

In two cases the results were negative as regards rise of temperature. One of them complained of headache and vertigo on the sixth day from the date of infection—temperature normal. The next day he complained of headache and pain at the site of inoculation. The symptoms cleared up on the third day. The other complained of headache and vertigo on the seventh day after infection—temperature 98.8°F—pulse 88—site of injection swollen and tender. Cleared up next day.

Chart 4

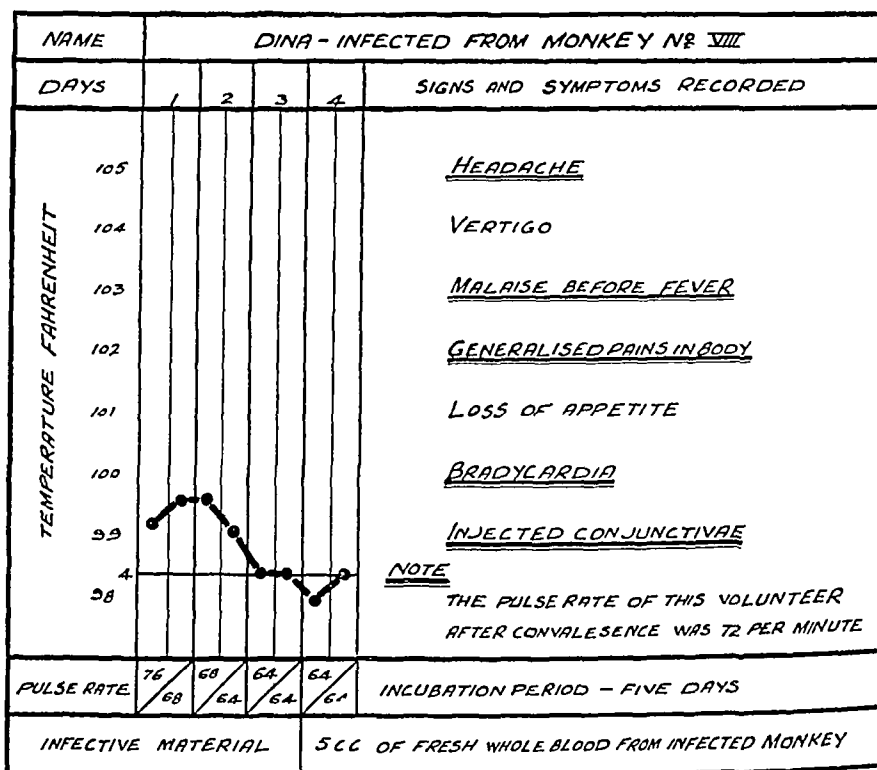


The temperature charts of monkeys infected by inoculation of human and monkey infective bloods

In the third case the volunteer contracted a definite, though modified, attack of sandfly fever as will be seen by a reference to Chart 5

Apart from the low temperature in this case the other signs and symptom of sandfly fever were quite typically manifested. In recording our findings in the human cases we adopted, at the beginning of our experiments, a *pro forma* which included all the signs and symptoms of sandfly fever described by various observers or seen in our own experience. A part of this *pro forma* is given with Chart 5

CHART 5



Sandfly fever induced in human volunteer by inoculation of infective monkey's blood

completed for the patient in question as an indication of the completeness of the evidence that the case really was a mild case of sandfly fever

In the *pro forma* the signs and symptoms which we consider most important in the diagnosis of sandfly fever are underlined and it will be seen that most of these were shown by the patient. The incubation period was five days and the duration of the fever three days. Malaise, headache and injected conjunctivae were marked symptoms. Extremely characteristic was the slow pulse which maintained a low level throughout the fever and for some days afterwards. The pulse rate of this individual has since returned to its normal rate of 72 per minute.

The fact that the attack was a comparatively mild one may have been due to the attenuation of the virus by passage through a monkey but, on the other hand,

it may have been due to a relative immunity, for several of our volunteers who were inoculated with infective human blood complained of malaise and vague muscle aches yet did not actually develop fever and so have been put down by us as failures in infection

To exclude the possibility that the inoculation into human subjects of normal monkey blood might produce the results recorded in these cases, two human controls were each inoculated in a manner similar to the volunteers with 5 c c of freshly drawn normal monkey blood. The temperature, pulse and symptoms were recorded daily for one week. There was no rise of temperature and the only complaint was slight tenderness at the site of inoculation on the day following the operation.

8 *No visible causative agent has been demonstrated in the blood by direct microscopical or cultural examination*

In all the typical cases of sandfly fever induced by us in Kasauli the blood was very carefully examined microscopically. With the exception of one case which will be mentioned in the next section no visible micro-organisms were encountered. The freshly drawn blood of six of the induced cases was cultured in Fletcher's medium. Six tubes of medium were inoculated from each case, about 0.75 c c of blood per tube being used. In no case was any causative micro-organism demonstrated and the cultures remained sterile.

9 *A Leptospira has been isolated from cases of so-called sandfly fever*

As stated in the last section the bloods of all the cases of sandfly fever induced at Kasauli were carefully examined. In one stained blood slide from volunteer No. 28 a single definite spirochætal organism was seen. Further slides taken from the same case failed to reveal any more organisms. Inoculation of blood into Fletcher's medium had been carried out at the same time at which the blood slide was made. These cultures remained sterile. It was thought that the most probable source of the single organism was the distilled water used in diluting the Leishman's stain used. A sample of the water in the bottle was centrifuged and the deposit stained and examined. Scrapings from the stopper of the bottle were similarly examined and the samples were also examined by darkground illumination. These examinations showed the presence of a variety of bacteria and, although no leptospira was noted, it is considered that the water was the probable source of the one specimen seen in the stained blood slide.

SUMMARY AND CONCLUSIONS

1 Blood specimens of sandfly fever cases from the Indian Frontier sent to Kasauli produced typical sandfly fever in human volunteers when inoculated subcutaneously.

2 Fresh blood from a locally induced case in Kasauli produced typical sandfly fever in another volunteer when inoculated subcutaneously.

3 Fresh whole blood, citrated whole blood, glycerinated whole blood, citrated glycerinated blood and filtrates of blood were all found capable of inducing the disease by subcutaneous inoculation.

4 Inoculation of infective human blood appears to have induced sandfly fever in monkeys

5 Inoculation of infective monkey's blood appears to have induced a mild but definite attack of sandfly fever in a human volunteer

6 Inoculation of infective monkey's blood appears to have induced sandfly fever in monkeys

7 The bites of sandflies fed on the Indian Frontier on cases of sandfly fever appear to have induced attacks of sandfly fever when fed upon volunteers in Kasauli

ACKNOWLEDGMENTS

In conclusion, we desire to express our thanks to Colonel E W C Bradfield, C I E , O B E , I M S , Assistant Director of Medical Services, Peshawar District, for the great interest taken and assistance given by him in this preliminary investigation. Our thanks are also due to Colonel I M Macrae, C I E , O B E , I M S , Officer Commanding, Indian Military Hospital, Peshawar, Lieut-Colonel A Campbell Munro, M D (Glasg), D P H , D T M (Camb) I M S , Officer Commanding, Indian Military Hospital, Landikotal, and Lieut-Colonel T H Scott, D S O , M C , M B , R A M C , Officer Commanding, British Military Hospital, Peshawar, for putting at our disposal all the clinical material required in the investigation.

To Captain A Sachs, M B , R A M C , we are greatly indebted for the laboratory facilities provided by him at the District Laboratory, Peshawar. It should be noted here, also, that it was due to investigations carried out by this officer in the previous year that the researches on sandfly fever were instituted by the Army Medical Authorities.

Dr I M Puri, M Sc (Punjab), Ph D (Cantab), F E S , who was working on the sandfly investigation under the Indian Research Fund Association, was of the greatest assistance to us and, by supplying us with laboratory-bred flies and arranging for the feeding of these on cases of sandfly fever, rendered possible the work on transmission by the bites of the flies.

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BACTERIOPHAGE IN THE TREATMENT AND
PREVENTION OF CHOLERA

A STATISTICAL EXAMINATION

PARTS I—IV

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BACTERIOPHAGE IN THE TREATMENT AND PREVENTION OF CHOLERA

A STATISTICAL EXAMINATION

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Part I

CHOLERA IN NOWGONG AND HABIGANJ

THE events which led to the trial of bacteriophage for the prevention of cholera by a method which is a corollary to the view advanced by d'Herelle (1926) that cholera epidemics are brought to an end by the dissemination of bacteriophage, began with a village epidemic of 143 cases of cholera (Morison and Pal Choudhury, 1930) This epidemic lasted 19 days On and after the tenth day bacteriophage was liberally used The mortality of the cases treated with bacteriophage dropped at once Fifty-one out of 57 cases treated with bacteriophage recovered and six untreated cases during the same period all died The graph of this epidemic, given on page 26 of a recent brochure (Morison, 1932), gives no evidence that bacteriophage shortened the epidemic even if we ascribe to the bacteriophage the dramatic change in the mortality, but, if the drop in the mortality was significant and not an accident, the graph suggested that the early administration of bacteriophage in villages, by the villagers themselves, might effect a material reduction in the mortality over areas, large enough to be statistically important, where cholera

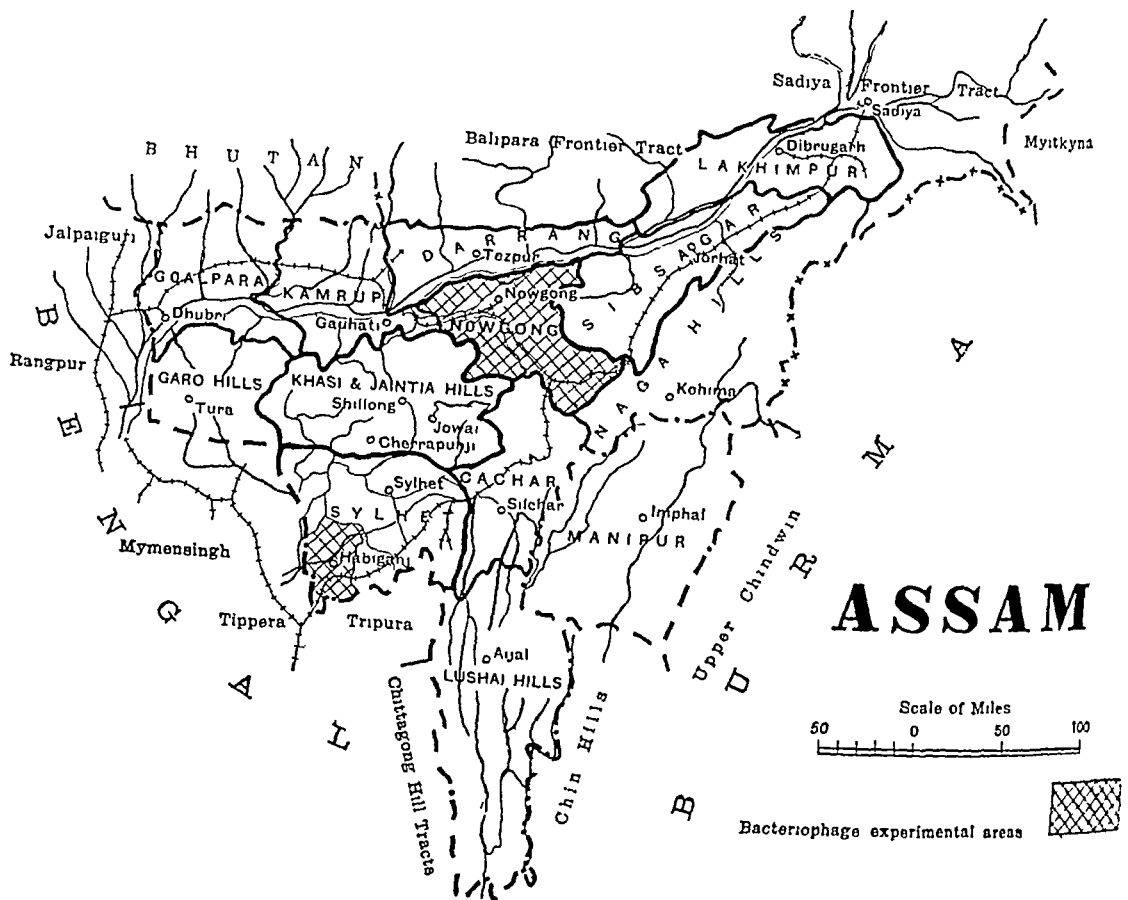
* For the services of Dr E M Rice, we are indebted to a grant from the Royal Society, London, and for those of Dr B K Pal Choudhury to the Indian Research Fund Association

was known to recur in epidemic form and from which cholera was hardly ever altogether absent

PREVENTION OF CHOLERA IN NOWGONG

Two areas were selected, the district of Nowgong, with a population of 562,581 and the Habiganj sub-division of Sylhet with a population of 632,521 (Map 1) Both areas have dying rivers along which it has been explained (Morison, 1932) cholera tends to appear, both are in the plains They are 140 miles apart, in different river basins The one is populated by Hindu Assamese

MAP 1



and by Bengali-Mussalman immigrants and the other mainly by settled Mussalman Sylhetis Both areas have adjoining districts also prone to cholera and suitable for controls

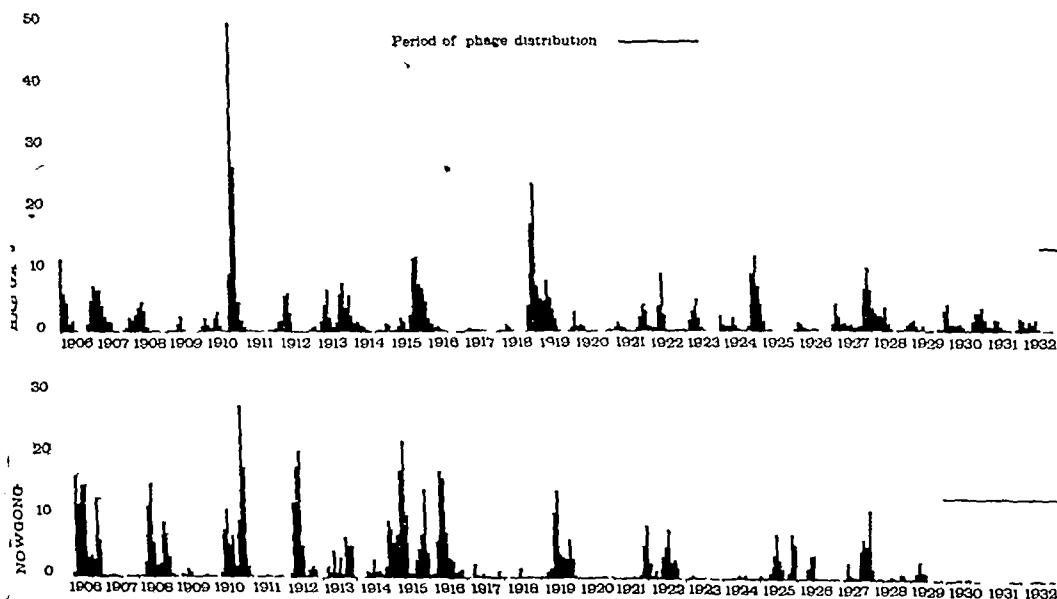
The graphs of the deaths of cholera in the two areas, already published in part (Morison, 1932), have been extended back to 1906, the earliest date to which the records in the office of the Public Health Department go, to make sure that the

prolonged absence of cholera from Nowgong has not happened before and both graphs have been brought up to December 1932* (Graph 1)

During 1932, the incidence of cholera throughout the Province of Assam was relatively low, but Nowgong passed through a severe test. Cholera was epidemic in the Kamrup, Darrang and Sibsagar districts, that is, on three sides of the Nowgong experimental area. Rail, river and road traffic constantly passes through these epidemic areas into the experimental area. In Kamrup, the district adjoining Nowgong on the west, there was, during the first nine months of the year, a series of outbreaks of cholera with 2,139 deaths. On 1st July, cholera was carried by a

GRAPH 1

DEATHS FROM CHOLERA PER 10000 OF POPULATION



Nepali travelling from Kamrup by railway through the Nowgong district to Sibsagar district, which adjoins Nowgong on the east. This man was removed from the train with cholera and died on 7th July, but he gave rise to 698 known cases with 268 deaths. Following this epidemic, minor outbreaks with 161 deaths took place in the Jorhat and Golaghat sub-divisions of Sibsagar along the Assam Trunk Road, westwards to Bokakhat, the nearest large village settlement to Nowgong district, 15 miles from it.

North of Nowgong is Darrang district, where, during September, October, November and December, there was an outbreak of 484 cases with 317 deaths

* Nowgong continued free from cholera during 1933

The focus of this epidemic was across the Brahmaputra and not more than five miles from Silghat. In Nowgong there is constant ferry traffic between the place where cholera was in Darrang and Silghat. Silghat is where the Kalang river leaves the Brahmaputra to flow through Nowgong district. It has been looked upon as the place where serious epidemics in Nowgong are most likely to arise.

Throughout the whole year, and under these conditions in the surrounding districts, there were only 27 deaths from cholera in Nowgong, a mortality of 0.46 per 10,000, the lowest mortality in the Province. Protective vaccination was wholly discontinued in Nowgong during 1932 and no special measures were taken other than the distribution of bacteriophage for diarrhoea, dysentery and suspected cholera. Weekly reports from the distributing staff of the Public Health Department, many of which have been checked in the villages themselves by our own staff, show that 3,906 cases of diarrhoea, dysentery and suspected cholera were treated with bacteriophage. Of all these only 52 cases died. The small number of deaths shows that bacteriophage is being largely used, as is intended, for mild cases as well as for the more severe.

Until June, Habiganj was the control area to Nowgong. There anti cholera vaccination had been energetically used together with propaganda and the treatment of cholera cases with essential oils or permanganate. Habiganj, during 1932, had the usual spring epidemic, when there were 474 deaths up till 30th June.

Habiganj was chosen in December 1929, as a control to Nowgong only because it was not possible, at that time, to use it as an experimental area. It has served its purpose. But we have always considered that Habiganj is well situated to be a parallel experiment to Nowgong and permission to use it as such was obtained in June 1932. At the end of June, the distribution of bacteriophage to the villages along the Barak river in Habiganj was begun and, to make the experiment definite, all vaccination was stopped, as it had already been stopped in Nowgong. From June 1932, therefore, we have two parallel experiments.

Considering the distribution of bacteriophage in Nowgong and Habiganj as two parallel experiments, the one beginning in December 1929, and the other in June 1932, we have to place beside each its appropriate controls. These controls are (1) the previous incidence of cholera in each area, (2) the incidence of cholera in similar areas in the same valley.

PREVIOUS INCIDENCE OF CHOLERA IN NOWGONG

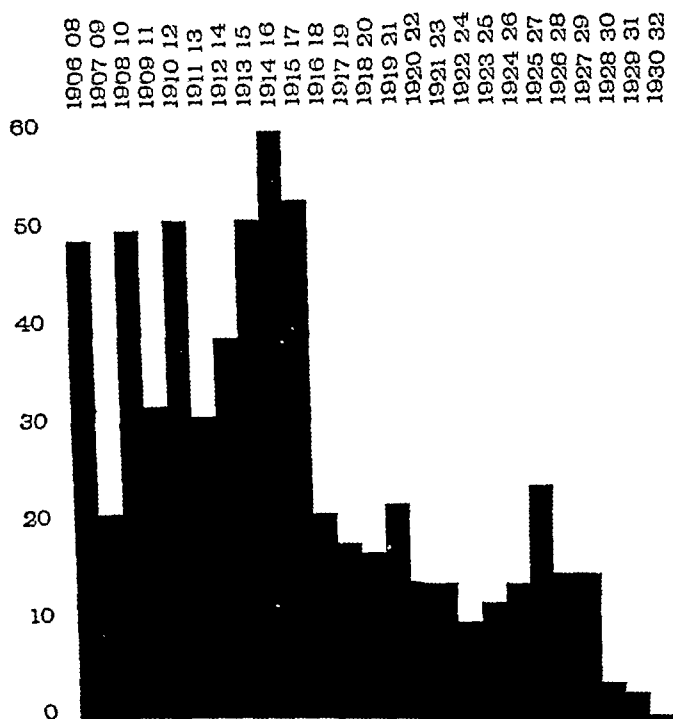
Since the experiment began in Nowgong three full years have elapsed. The deaths per 10,000 of the population have been for 1930, 0.99, for 1931, 0.78 and for 1932, 0.46*. The death rates month by month since January 1906 have been calculated (Table XXXVII—see Appendix), the three lowest yearly death rates between 1906 and 1929 were 1.34 in 1918, 1.42 in 1920 and 1.62 in 1923. There has been no period of three consecutive years previous to 1930 in which the death rates have been comparable to those of 1930, 1931 and 1932. The period of three years covers six epidemic seasons, for cholera is liable to occur between March and June and again between September and January. It is sufficiently long to compare it with a 'moving average' of cholera deaths during every three-yearly period since

* For 1933 the death rate from cholera was 0.36

1906 This is plotted in Graph 2 The graph is sharply divided into three periods a period of severe incidence, 1906-1919, a period of moderate incidence, 1920-1929, and a period, 1930-1932, in which there was little cholera culminating in the full three-yearly period of the experiment

GRAPH 2
CHOLERA in NOWGONG

Moving Average of deaths per 10000 of population for
three yearly periods



After March 1919 every immigrant coolie to the Assam tea-gardens recruited by the Tea District Labour Association was vaccinated before he left the recruiting depôts throughout India for the journey, under crowded conditions by rail and steamer, of three to ten days to his tea-garden, a journey during which cholera was notoriously liable to occur. The compulsory vaccination of immigrants, of whom from 22,000 to 92,000 come every year to Assam, evidently hindered the importation of cholera but did not materially affect the endemic cholera of the villages

where reliance was placed on propaganda and vaccination after news of an epidemic had been received

INCIDENCE OF CHOLERA IN CONTROL AREAS

In the Brahmaputra Valley, in which Nowgong lies, the other districts are Goalpara, Kamrup, Darrang, Sibsagar and Lakhimpur. In two important respects, Sibsagar and Lakhimpur differ from the other districts in the valley. In the first place, in neither Sibsagar nor Lakhimpur are there drying rivers along which epidemics spread with facility, as are met with in the other districts. In the second place, tea-garden labour forms a large proportion of their population. In 1919, when, owing to the famine and distress caused by influenza, labour flowed freely to Assam, there was a severe epidemic of cholera in Lower Bengal through which these immigrant labourers passed by river or rail to Assam. Large numbers fell ill on the way and brought cholera with them to the tea-gardens. The gardens of Sibsagar and Lakhimpur were particularly affected. The adoption of compulsory vaccination for immigrant labour affected beneficially the tea districts. The districts most concerned with the importation of labour, Sibsagar and Lakhimpur, exhibit, from 1920 onwards, a graph for cholera which is a striking contrast to that of the preceding years from 1906 (Graph 3, Tables XXXVIII and XXXIX—see Appendix). Vaccination of immigrant labour before that labour left the recruiting depôts in different parts of India lessened the importation of cholera. There was no widespread vaccination in the tea-gardens themselves. It is the importation of infection that is guarded against. This, of course, is not applicable to villages which preponderate in the districts of the Brahmaputra Valley other than Lakhimpur and Sibsagar. For these two reasons neither Sibsagar nor Lakhimpur are suitable controls to Nowgong.

Of the remaining three districts, Kamrup, Goalpara or Darrang, it would appear from Graph 3 and Tables XXXIV, XXXV and XXXVI (see Appendix) that any one may be taken as a control. Darrang which adjoins Nowgong on the opposite bank of the Brahmaputra seems the most appropriate, but, as far as we can judge, the choice is immaterial.

We have examined the correlation for the deaths per 10,000 of the population month by month in Nowgong and Darrang from 1906 to 1929, that is for 288 months (Table I). The correlation is $+0.611 \pm 0.037$. The significance of this has been tested by the calculation of ' t ', $t = 13.062$ and $p =$ less than 0.01, that is to say, that the probability of this correlation occurring by chance in this period is less than 1 in 100 (Fisher, 1930). With such a correlation coefficient and with the knowledge that the districts are similar in climate, rainfall, and, for the greater part, in soil, the two areas are as similar as any two areas of 3,896 square miles and 2,842 square miles are likely to be.

We consider epidemic cholera to have broken out in a district when the deaths from cholera in any month are more than 0.5 per 10,000 of the population. The population of Darrang being 355,077 in 1906 and 596,833 in 1932, a death rate of 0.5 per 10,000 is equivalent to about 25 deaths from cholera. Under this number, the cases are considered sporadic and the diagnosis may be open to question. When over this number, an inspection of the records shows definitely localized outbreaks.

GRAPH 3

DEATHS FROM CHOLERA PER 10000 OF POPULATION

BRAHMAPUTRA VALLEY

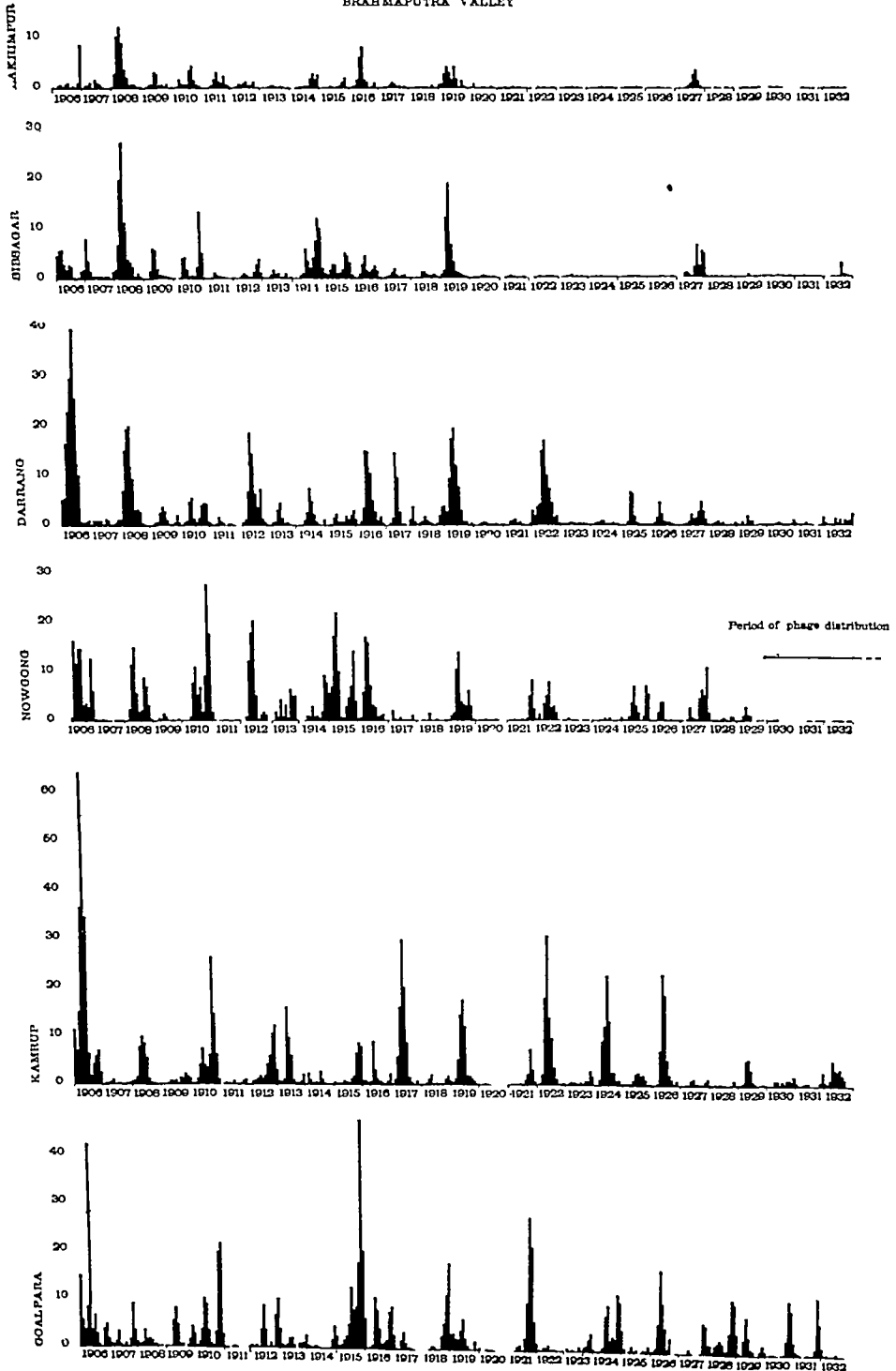


TABLE I
Correlation between death rates in Nongong and in Darrang, 1906-1929

Nowgong				DARRANG				
Deviation	Frequency	Deviation × frequency	Deviation-× frequency	Frequency	Deviation × frequency	Deviation²× frequency	Total for Nowgong	Product
-2.25	158	-355.5	799.875	110	-247.5	556.875	-163.25	367.3125
-1.75	28	-49.0	85.75	62	-108.5	189.875	-64.50	112.1750
-1.0	22	-22.0	22.0	38	-38.0	38.0	6.0	-6.0
0	9	-426.5		18	-394.0		13.0	
1.0	16	16.0	16.0	12	12.0	12.0	10.75	10.75
2.0	4	8.0	16.0	11	22.0	44.0	44.25	88.5
3.0	11	33.0	99.0	4	12.0	36.0	4.5	13.5
4.0	6	24.0	96.0	3	12.0	48.0	18.75	75.0
5.0	8	40.0	200.0	6	30.0	150.0	0.75	3.75
6.0	1	6.0	36.0	0	0	0	0	0
7.0	3	21.0	147.0	3	21.0	147.0	0.25	1.75
8.0	3	24.0	192.0	3	24.0	192.0	7.0	56.0
9.0	3	27.0	243.0	0	0	0	0	0

10 0	2	20 0	200 0	1	10 0	100 0	12 0	120 0
11 0	0	0	0	0	0	0	0	0
12 0	4	18 0	570 0	2	21 0	238 0	0 75	0 0
13 0	1	13 0	160 0	1	52 0	670 0	20 0	200 0
14 0	2	28 0	392 0	1	11 0	100 0	-1 75	-21 5
15 0	3	15 0	675 0	2	30 0	450 0	2 0	30 0
16 0	1	10 0	250 0	1	16 0	250 0	22 5	300 0
17 0	0	0	0	1	17 0	280 0	9 0	153 0
22 5	3	67 5	1,518 75	5	112 5	2,531 25	58 0	1,200 0
32 5	0	0	0	1	32 5	1,050 25	12 0	1,010 0
TOTALS	298	136 5 - 126 5	5,730 375	288	412 0 - 394 0	7,250 25	239 5 - 220 5	3,030 2375
		10 0	5,739 375		48 0	7,250 25	10 0	3,930 2375
	Correction for mean —		0 3472 5,739 0278	Correction for mean —		8 0 7,248 25	Correction for mean —	1 0666 3,928 5709
	Sheppard's correction —		24 0 5,715 0278	Sheppard's correction —		24 0 7,221 25		

$$r = \frac{3928.5709}{\sqrt{5715.0278 \times 7524.25}} = +0.6114 \quad \pm 0.0369 \quad t = 13.0623 \quad p = \text{much less than } 0.01$$

TABLE II

Correlation between death rates in Nougong and in Darrang, 1930-1932

NOWGONG			DARRANG			
Deaths per 10,000	Deviation from mean (x)	Deviation-	Deaths per 10,000	Deviation from mean (y)	Deviation*	Product (xy)
0 11	0 034	0 001156	0 05	-0 4464	0 19927	-0 01618
0 09	0 014	0 000196	0 33	-0 1664	0 02769	-0 00233
0 06	-0 016	0 000256	0 05	-0 4464	0 19927	0 00714
0 11	0 034	0 001156	0 16	-0 3364	0 11316	-0 01144
0 35	0 274	0 075076	0 37	-0 1264	0 01598	-0 03463
0 00	-0 076	0 005776	0 52	0 0236	0 00056	-0 00179
0 22	0 144	0 020736	0 21	-0 2864	0 08202	-0 03404
0 40	0 324	0 104976	0 17	-0 3264	0 10654	-0 10375
0 00	-0 076	0 005776	0 02	-0 4764	0 22696	0 03621
0 09	0 014	0 000196	0 19	-0 3064	0 09348	-0 00429
0 07	-0 006	0 000036	0 21	-0 2864	0 08202	0 00172
0 00	-0 076	0 005776	1 10	0 6036	0 36433	-0 04587
0 00	-0 076	0 005776	0 42	-0 0764	0 00584	0 00581
0 00	-0 076	0 005776	0 12	-0 3764	0 14168	0 02861
0 12	0 044	0 001936	0 24	-0 2564	0 06574	-0 01128
0 00	-0 076	0 005776	0 17	-0 3264	0 10654	0 02481
0 07	-0 006	0 000036	0 67	0 1736	0 03014	-0 00104
0 02	-0 056	0 003136	0 10	-0 3964	0 15713	0 02220
0 02	-0 056	0 003136	0 29	-0 2064	0 04260	0 01156
0 00	-0 076	0 005776	0 03	-0 4664	0 21753	0 03545
0 04	-0 036	0 001296	0 00	-0 4964	0 24641	0 01787
0 21	0 134	0 017956	0 07	-0 4264	0 18182	-0 05714
0 14	0 064	0 004096	0 17	-0 3264	0 10654	-0 02089
0 16	0 084	0 007056	1 64	1 1436	1 30782	0 09606
0 00	-0 076	0 005776	0 69	0 1936	0 03748	-0 01471
0 02	-0 056	0 003136	0 20	-0 2964	0 08702	0 01660
0 00	-0 076	0 005776	0 30	-0 1964	0 03857	0 01493
0 03	-0 046	0 002116	0 32	-0 1764	0 03112	0 00811
0 07	-0 006	0 000036	1 49	0 9936	0 98724	-0 00496
0 09	0 014	0 000196	0 55	0 0536	0 00287	0 00075
0 05	-0 025	0 000625	1 36	0 8636	0 74580	-0 02245
0 05	-0 026	0 000676	5 35	-0 1464	0 02143	0 00381
0 00	-0 076	0 005776	1 01	0 5136	0 26378	-0 03903
0 12	0 044	0 001936	0 92	0 4236	0 17944	0 01779
0 03	-0 046	0 002116	1 02	0 5236	0 27416	-0 02409
0 00	-0 076	0 005776	2 36	1 8636	3 47300	-0 14163
2 74	1 222	0 316856	17 87	7 374	10 26338	0 34943
.	-1 218	0 0088		-7 374	0 2851	-0 59354
						-0 24111

Mean

0 0761

$$r = -0.1354 \pm 0.11$$

$$t = 0.7969$$

$$p = 0.4$$

The correlation coefficient derived from this table is insignificant

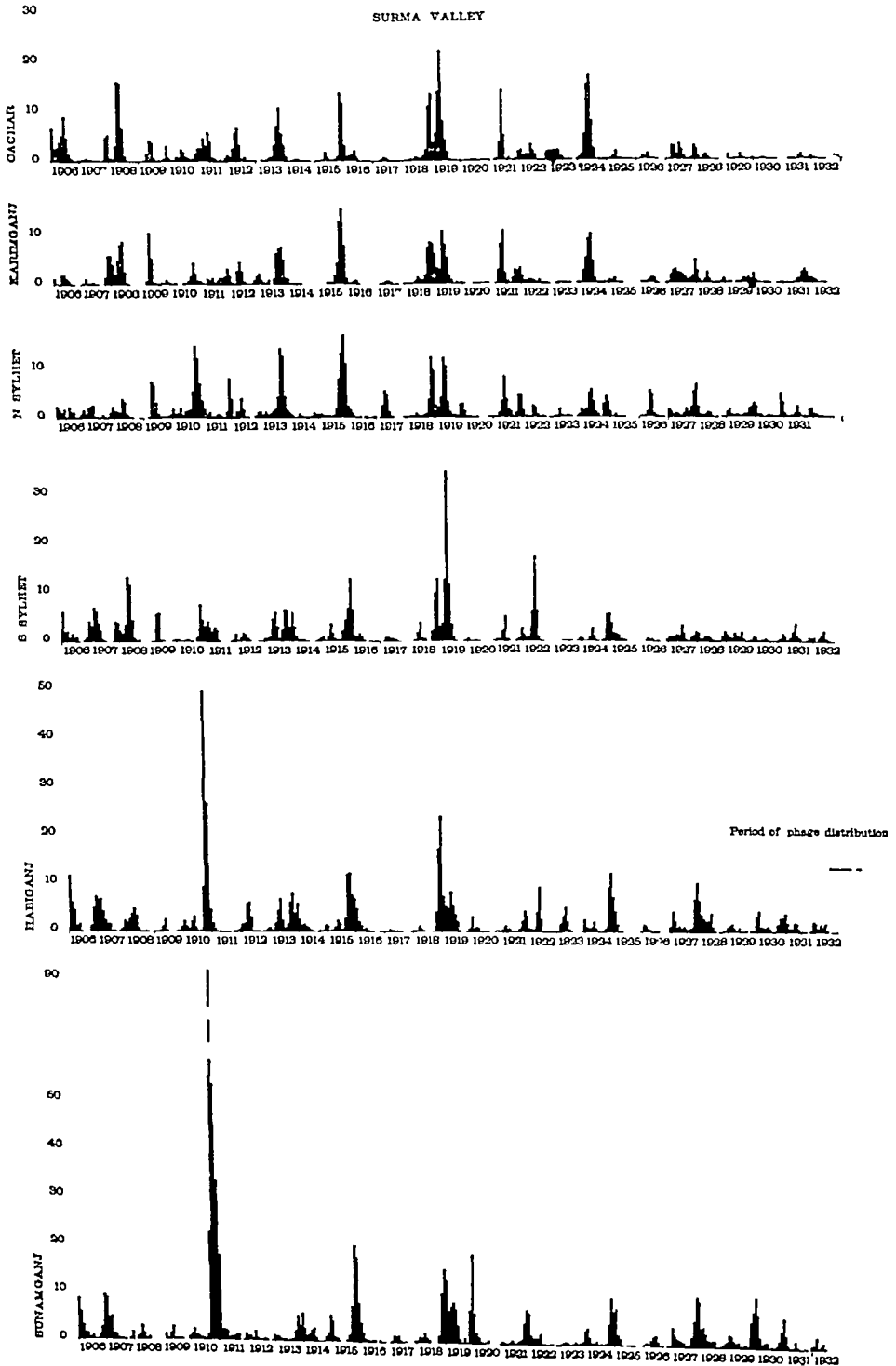
Mean

0 4964

GRAPH 4

DEATHS FROM CHOLERA PER 10000 OF POPULATION

SURMA VALLEY



Returning now to the period 1906-1929, Darrang had a death rate above 0.5 in 178 months out of a total of 288 months. The probability of any one month having more than 0.5 deaths per 10,000 was $\frac{178}{288}$ or 0.618. We might, therefore, expect more than 0.5 deaths per 10,000 in about 22 of the 36 succeeding months, the actual number was 12.

In Nowgong from 1906 to 1929 there were 130 months out of 288 in which the deaths from cholera exceeded 0.5 per 10,000. The probability of any one month having more than 0.5 deaths per 10,000 was $\frac{130}{288}$ or 0.451. During the next 41 months January 1930-May 1933 we might have expected 18 months to have had more than 0.5 deaths per 10,000, actually there were none.

If a common cause had reduced the cholera in Nowgong and in Darrang during the last three years, but had done so to a much greater extent in the former than in the latter, it is probable that there should still remain a correlation between cholera deaths in both areas. We have worked out this correlation, $r = -0.1$ in contrast to $r = +0.6$ for the previous 288 months (Table II)*.

It is, therefore, apparent that whether we take as a control for Nowgong the distant area of Habiganj, the previous history of Nowgong itself or the adjacent area of Darrang the result is the same. Something has happened in Nowgong which has not happened before and has not happened in the control areas.

In Habiganj the experiment began in July 1932, and in our graph (Graph 4, Tables XL to XLV—see Appendix) we have set forth the previous history of this and of the adjoining areas of North Sylhet, South Sylhet, Sunamganj, Karimganj and Cachar. Cachar, like Lakhimpur, has many tea-gardens but the other areas are fair controls to Habiganj, provided that Habiganj is not, as there is now reason to suspect, a source from which the other districts are infected.

The stage is set for this second experiment. Both experiments, we believe, meet, as far as is practicable in Assam, the suggestion kindly made to us by Professor Greenwood, F.R.S., that the country should be divided as in an agricultural test. We have in the Brahmaputra Valley four 'plots' as nearly as possible similar in natural features, climate and race and, in the Surma Valley (Sylhet and Cachar), we have six plots again similar. From each valley we selected one plot for experiment for no other reason than that it was notoriously liable to cholera. When funds are available we propose to extend the experiment to plot after plot.

DISTRIBUTION ORGANIZATION

It may be advisable to explain the organization of the village distribution. Assam is divided into eight 'plains' districts. The 'hill' districts do not concern us for they are only invaded by cholera from the plains. The districts vary in size

* 'The correlation of 0.61 shows that there was a similarity in the course of the mortality in Nowgong and Darrang. The existence of this correlation coupled with the fact that in the 36 months of the experiment there was no significant correlation does seem to provide evidence that bacteriophage treatment was having an effect' (A. B.)

from 2,842 square miles to 5,478 square miles The populations vary from 562,581 to 2,724,342 (Table III)

TABLE III

District or sub division		Area, square miles	Number of villages	Population	
<i>Brahmaputra Valley —</i>					
	Goalpara	3,985	3,188	882,748	
	Kamrup	3,844	2,738	976,746	
	Darrang	2,842	1,978	584,817	
	Nowgong	3,898	2,323	562,581	
	Sibsagar	5,131	2,284	933,326	
	Lakhimpur	4,234	2,498	724,582	
<i>Surma Valley —</i>					
Sylhet	North Sylhet	1,090	2,551	580,560	2,724,342
	South Sylhet	865	2,032	451,364	
	Habiganj	1,003	2,530	632,521	
	Sunamganj	1,443	2,788	550,263	
	Karimganj	1,077	1,816	509,634	
	Cachar	3,862	1,607	570,531	
TOTALS		33,274	28,333	7,959,673	

In the Brahmaputra Valley the districts are divided into sub-divisions. Each sub-division is divided into thanas or police areas, each thana, except in Goalpara which follows the practice of Surma Valley districts, into mauzas under a mauzadar. A mauza includes a group of from 14 to 103 villages. Every village has a gaonbura or headman in charge of 120 to 150 houses. In Nowgong, for the purpose of distribution to its 2,323 villages, the mauzas were divided into nine circles to each of which was allotted an assistant distributor with a sub-assistant surgeon in charge of three circles. These visited, in rotation, all the villages in their circles explaining the use of bacteriophage and replenishing the stock of each gaonbura as occasion required. Between the visits of the distributors a gaonbura can replenish his stock from the reserve kept by the mauzadar when the former appears each month before the mauzadar with his register of births and deaths.

In the Surma Valley the arrangements are somewhat different. Sylhet, one of the largest districts in India, is divided into five sub-divisions each of which in size and population is comparable to a whole district in the Brahmaputra Valley.

The sub-divisions are divided into thanas and the thanas into administrative circles each in charge of a surpanch who has four assistants (panches). The circles are further divided into beats, each with a chaukidar who is responsible for watch and ward. Each of these chaukidars has charge of about 80 houses. He records births and deaths and any other occurrence in books and produces these books at the chaukidari parade at thana headquarters held once a week, once a fortnight or once a month, depending on the distance of the beat from the thana. Adjoining each thana headquarters is a dispensary where bacteriophage is stocked. In Habiganj the distribution is made to 2,530 villages by four sub-assistant surgeons and six assistant distributors in circumscribed areas similar to those in Nowgong. The chaukidars, when they come to thana headquarters with other reports, have also to report how much bacteriophage they have in their possession, and, if necessary, to replenish their supply from the adjacent dispensary.

In both Nowgong and Habiganj, the arrangements for distribution are under the charge of the civil surgeon, whose staff, as well as the staff of the Pasteur Institute, make a periodic check to ensure a constant supply of bacteriophage to every village at all times. Each gaonbura or chaukidar keeps in his possession one box containing 16 ampules of bacteriophage for every 125 persons in his village. In both areas all distributors are given stamped and addressed post cards which are filled up as each village is visited. We aim at one sub-assistant surgeon or distributor visiting each village at least once in two months. These cards give the survey number of the village visited so that when they are filed according to these numbers it is easy to see if any village is missed. The cards also record the amount of bacteriophage stock at the time of the visit, the amount supplied, and the amount used since the last visit. A register kept by the gaonbura or chaukidar shows the number of cases of dysentery, diarrhoea or suspected cholera which have occurred since the last visit. This information is all transferred to the post card so that we are in a position to know should there be any increase in sickness or any omission in distribution. Both local boards devote their grant for cholera to the bacteriophage experiment and when the grant for the year is exhausted the balance required by the board is supplied by the Public Health Department or by a special grant by the Government.

Part II

DISTRICT EPIDEMICS

Between July and December 1932, there occurred four cholera epidemics in the districts of Sibsagar and Darrang

The first (Sibsagar epidemic) occurred along a river bank where the river was the source of water-supply, the second was among estate labourers (Darrang tea estates, group 'A'), where the water-supply was from wells. The third was in scattered villages (Darrang villages) where the water-supply was principally from wells, while the fourth was among another group of estate labourers (Darrang tea estates, group 'B') where the water-supply was, in different gardens, from a river wells and a piped water-supply. Each epidemic had its characteristics

Every affected house in these four epidemics of nearly 1,200 cases was visited and each surviving patient or the relatives of the dead were seen. The data collected are, we believe, as accurate as it is possible to obtain under rural conditions

In the first of these epidemics bacteriophage was used on a fairly large scale for treatment (247 out of 676 cases for which the data are complete). In the third epidemic bacteriophage was used in 117 out of 308 cases. In the second and fourth no bacteriophage was used except for one case, other forms of treatment being employed. Other forms of treatment were also used for smaller numbers in the third epidemic

Before giving the mortality and the data relating to the infectivity in these epidemics, it is necessary to give a general account of each

SIBSAGAR EPIDEMIC

An epidemic of cholera resulting in 699 cases with 268 deaths broke out in the Sibsagar sub-division of Assam along the banks of the Dikhu river in July 1932 and continued through the months of August and September. The epidemic apparently began spontaneously in an area which had not been visited by cholera since 1917 and it was the first large epidemic in Assam for which bacteriophage had been used by the Medical and Public Health Departments. In smaller epidemics in Jakrem (Morison and Pal Chaudhury, 1930), Shillong, and Salmara (Pasteur Institute Report 1929), previously reported, bacteriophage treatment had been carried out by members of our own staff

The Sibsagar sub-division is in the Brahmaputra Valley in the north-eastern corner of India. It lies between the Naga Hills on the south and east and the Brahmaputra river on the north and west. The Dikhu river, on the banks of which the epidemic occurred, takes its origin in the Naga Hills and passes through the Sibsagar sub-division to join the Brahmaputra. During the rains, from June to October, the Dikhu is navigable for river launches, barges and small paddle steamers, the current is swift and the river may rise and fall as much as fifteen feet in 24 hours. During the dry weather the river is fordable in many

places and the current is slow. In its lower reaches the river level is largely dependent upon the height of the Brahmaputra which, when in flood, flows back into the Dikhu and, during this epidemic, was observed to slow up the flow of the Dikhu near its mouth.

The country through which the Dikhu flows after it leaves the Naga Hills is a flat alluvial plain. The banks of the river are raised to form large bunds, dykes or levees. These are the highest land in this part of the country and protect the surrounding low-lying land from flooding. It is along the broad banks of the river that most of the villages are placed. Few houses are more than 200 yards from the river. None of the villages have wells.

The villages visited by cholera are inhabited principally by fishermen (Kaibartas), Hindus of the Koch, Kalita and Keot castes, a few Ahoms and Mohammedans. The following table gives the population and religions ascertained at the 1931 census (Table IV). Here the term 'Hindu' includes the above castes and often Ahoms. In our records we have the caste of each person affected as given by a member of the family for, in this particular epidemic, caste had an overwhelming influence in determining the spread and limitations of the epidemic.

TABLE IV

Name of village	Number of houses	POPULATION			
		Hindu	Mohammedan	Others	TOTALS
Hansara	57	300	4		304
Julagaon	79	422	63		485
Noali Christiangaon	135	103	625	34	762
Sibsagar Town	1,269	4,490	2,012	167	6,669
Arjunguri .	76	338	55		393
Phulpanisega	106	480			480
Desial (Meteka)	145	622	189		811
Mathadang .	123	714			714
Kamarphodia	141	662	121		783
Senchuagaon .	85	197	205		102
Chaulkara .	219	952	337		1,289
Karigaon ..	227	1,268	17		1,285
Baliaghat ..	206	823	379	5	1,207

TABLE IV—concl'd

Name of village	Number of houses	POPULATION			
		Hindu	Mohammedan	Others	TOTALS
Pathualal	100	529	10		539
Fakum	166	915	16		931
Pathualal (Namdang)	131	645	3	1	649
Namdang Kumargaon	57	291			294
Phukenphodia	229	1,033	118		1,151
Namdang Bangalgaon	149	859			859
Desial (Jagichuk)	116	786	4		790
Kharedhara	88	631	2		633
Gatonga	8	47			47
Mugla Pukri	6	10	6		16
Bharaluagaon	114	696			696
Goalgyangaon	166	907	16		923
Morisuti	85	417	90		507

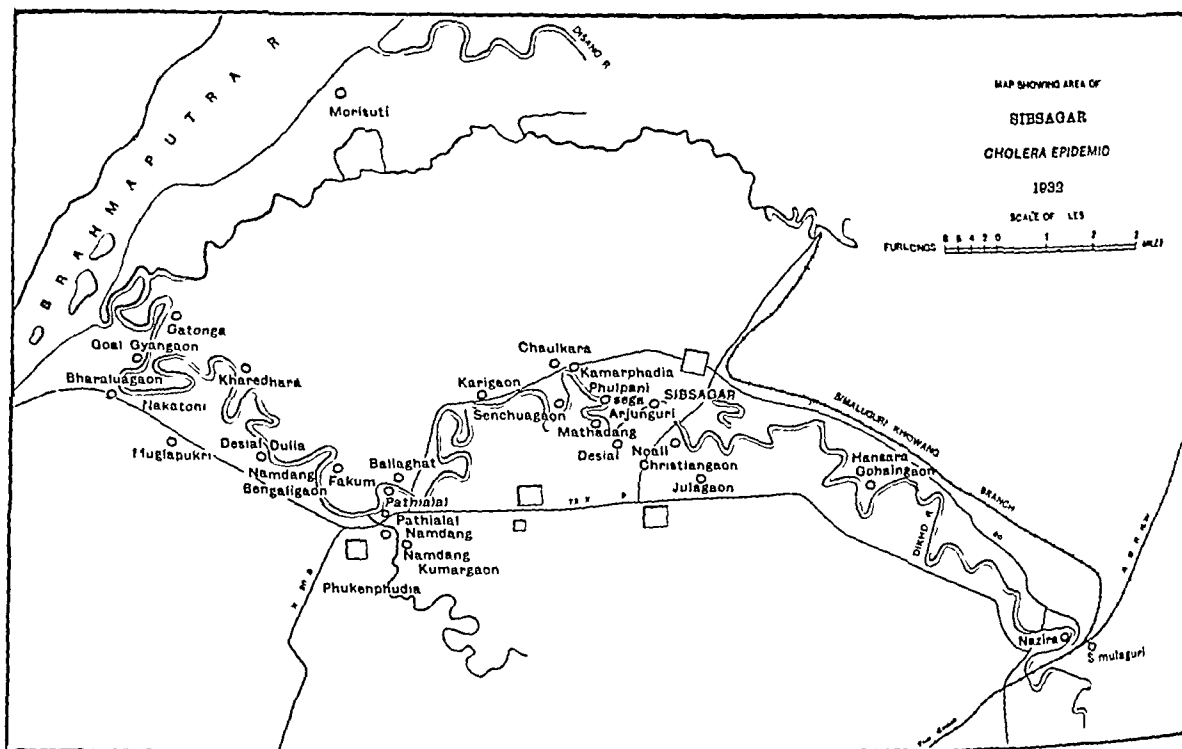
In this area communications are slow. The first intimation of the epidemic (at Pathualal) reached the town of Sibsagar, about six miles away, on 29th July. By that time 17 villages had been infected and in about half that number cholera was rapidly spreading. Before that date, bacteriophage had been sent to two villages, Pathualal and Namdang, for severe cases of diarrhoea not recognized by the villagers as cholera. During the first week of August the assistant surgeon and four sub-assistant surgeons at Sibsagar attended the sick within reach and began a vaccination campaign. Between 10th and 13th August these were reinforced by three more sub-assistant surgeons and six local practitioners and, between the 17th and 19th, by two more sub-assistant surgeons, the Assistant Director of Public Health and another assistant surgeon. This staff carried out energetically anti-cholera vaccination, distribution of bacteriophage and advised the people how to avoid cholera.

Our investigation began on 13th August when the epidemic was nearing its close. Every house was visited at least twice, usually by a different investigator on each occasion. One of us (E M R or B K P C) saw each surviving patient or the relatives of the dead. Eight weeks were occupied in collecting and verifying the data in the villages.

The fixing of dates is a serious difficulty in villages where none is literate and where few or no calendars exist, but we were assisted by the occurrence of a flood of the Dikhu river on 27th and 28th July, by a serious earthquake which happened on 14th August and, in one or two important instances, by the noting of the dates of deaths by the Brahmin responsible for fixing subsequent ceremonies. In a few cases the dates were given by the school master of the village and, in all cases, they were checked by reference from one house to another.

The origin of the epidemic was traced to a man who, travelling from Behar, stopped a day and a night (29th-30th June) at Gauhati where cholera was present

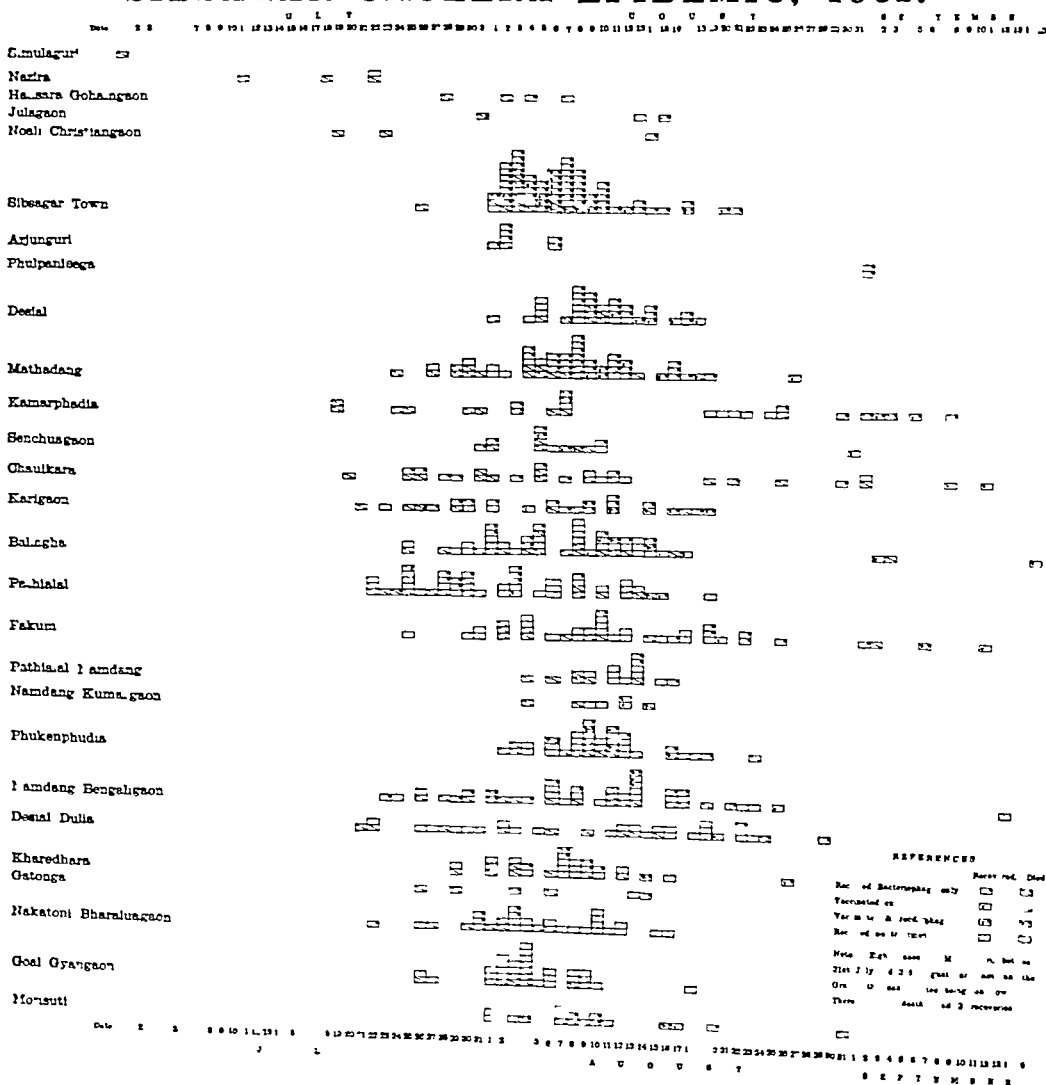
MAP 2



Actual primary infections from the river were not more than 32. These occurred between 19th and 27th July, when the river was flowing slowly and were

GRAPH 5

SIBSAGAR CHOLERA EPIDEMIC, 1932.



earlier in the higher villages. On 27th July the river began to rise rapidly owing to heavy falls of rain in the Naga Hills and had risen 18 feet by the 29th. Subsequent infections in the villages followed commensual lines and contrasted

with the water-borne infections which affected different castes indiscriminately. After the 27th we have no record of cases that could not be readily explained as commensal infections. Primary water-borne cases were scattered at random in houses along both sides of the river and, from these foci, the disease spread from inmate to inmate, from house to house and, in a few instances, was traced from village to village. House-to-house and village-to-village infections were facilitated by 'Amata' and other ceremonies which were held for the recovery of the sick. At these ceremonies the people of the community of about the same age as the patient were called together and fed.

DARRANG TEA ESTATES, GROUP 'A' EPIDEMIC

The epidemic considered under this group consists of one large garden (M tea estate), its out-garden (K tea estate) and a small village located midway and about a mile from each of the gardens. The inhabitants of the village were employed on the estates supplementing the estate labour.

M tea estate is situated on the north bank of the Brahmaputra on a flat alluvial plain. K tea estate is further removed from the Brahmaputra and about two miles from the main garden. Water-supplies for both estates and for the village are from surface wells adjacent to the houses, most of which are lined with brick or concrete rings but are unprotected at the top. Each individual draws water with his own bucket or earthenware vessel. No water is taken from the Brahmaputra but labourers do, occasionally, go to the river to bathe and fish. No latrines are used, the soil around the houses and land adjacent to the lines being used for latrine purposes.

In both estate lines, the houses, 20 to 40 feet apart, are arranged in streets with little or no fencing to separate the houses one from another. The caste, and hence customs, of the labourers are many but the caste system here does not apply to water-supply, all taking water from the common wells distributed in the lines. There are no common feeding centres, each family cooks its own food. There is a common bazaar within 400 yards of the M tea estate lines, it is frequented by labourers from both estates, by many villagers in the vicinity and also by shop-keepers from other areas. This bazaar is held once a week on Sundays.

Prior to 13th September, i.e., when the first case of cholera appeared in M tea estate, there had been a case in a Nepali house near the river steamer station, one and a half miles distant. This case had been to the M tea estate bazaar to sell milk. The two original cases on the estate had not visited the house of the Nepali. The relatives of the Nepali case near the river some times herd their buffaloes across the Brahmaputra near to Bokakhat, where cholera was known to be present—an extension from the Sibsagar epidemic. There was daily river traffic to the steamer station from both Kamrup and the Sibsagar districts. This primary case might have been infected from Bokakhat, from Kamrup or from Sibsagar, we were not able to determine which.

Both of the first two cases on M tea estate were removed to the estate hospital as soon as they were reported and both received hypertonic saline treatment. Both recovered, were discharged on 21st September, and they returned to their houses.

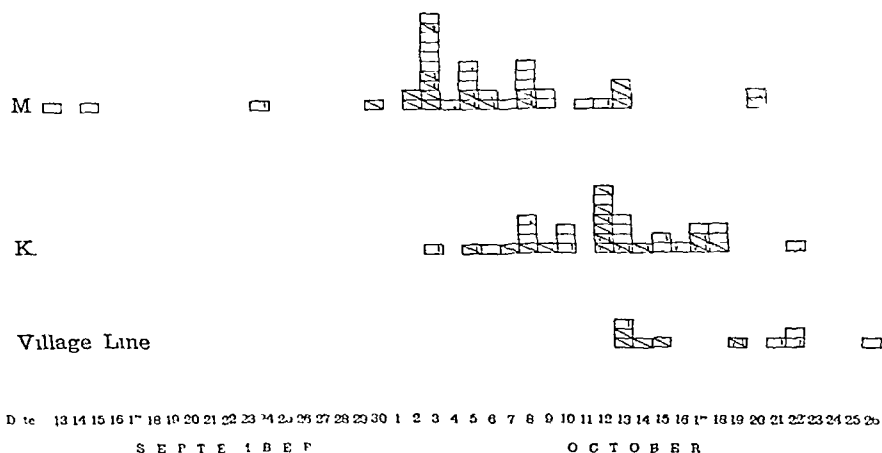
which were some distance from one another. Subsequent to their discharge two other cases developed on the 24th and 30th September and on 2nd October the rise in the incidence occurred (Graph 6). As each case was reported it was immediately removed to hospital.

The house of the case occurring on 15th September took its water-supply from a well only 25 to 30 yards away and, subsequent to the return of this case from hospital on 21st September, 14 other labourers, who also took their water from the same well, developed cholera. The first two of these cases occurred in one

GRAPH 6

CHOLERA in DARRANG Tea Estates 1932

Group A



REFERENCES

	Recovered	Died
Hypertonic Saline	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Permanganate	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Essential Oils	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Bacteriophage	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Vaccinated	<input type="checkbox"/>	<input checked="" type="checkbox"/>
No treatment	<input type="checkbox"/>	<input type="checkbox"/>

house, the remaining twelve cases in separate houses, a distribution characteristic of water-borne cholera.

Similarly, the case which occurred on 13th September returned to her house on the 21st September and drew water from another adjacent well. Here, among those using the same well-water, 18 labourers were infected, eight of these being in four houses and the remaining ten in separate houses. No case occurred in areas supplied by other wells in the same neighbourhood. These are two instances of convalescents from a hospital starting epidemics. No bacteriophage had been used.

The first case in the K tea estate occurred on Monday, 31d October, one day after the general bazaar on the M tea estate to which most of the labourers of the former estate go weekly for their supplies. Subsequent cases occurred between 5th and 22nd October, all being in the lines close to the centre of the garden and supplied by a few wells. Of the subsequent cases three were in one house, six others in three houses and the balance 24 each in a separate house.

In the village, the first three cases occurred on 13th October in the one house, being a man and his two children, the father had worked on the M tea estate the previous day. Subsequent cases followed between the 14th and the 26th, two being in one house and the remaining five in single houses.

Each case, from both estates and the village, was removed to the M tea estate hospital at once, the house occupied by the case was as effectively 'disinfected' as possible. As soon as vaccine could be obtained every worker was vaccinated but vaccination was not started before the 10th. Vaccination of the labour force took several days. Water in the wells was treated with chlorine solution and the soil in the lines was turned over by hoeing.

DARRANG VILLAGES EPIDEMIC

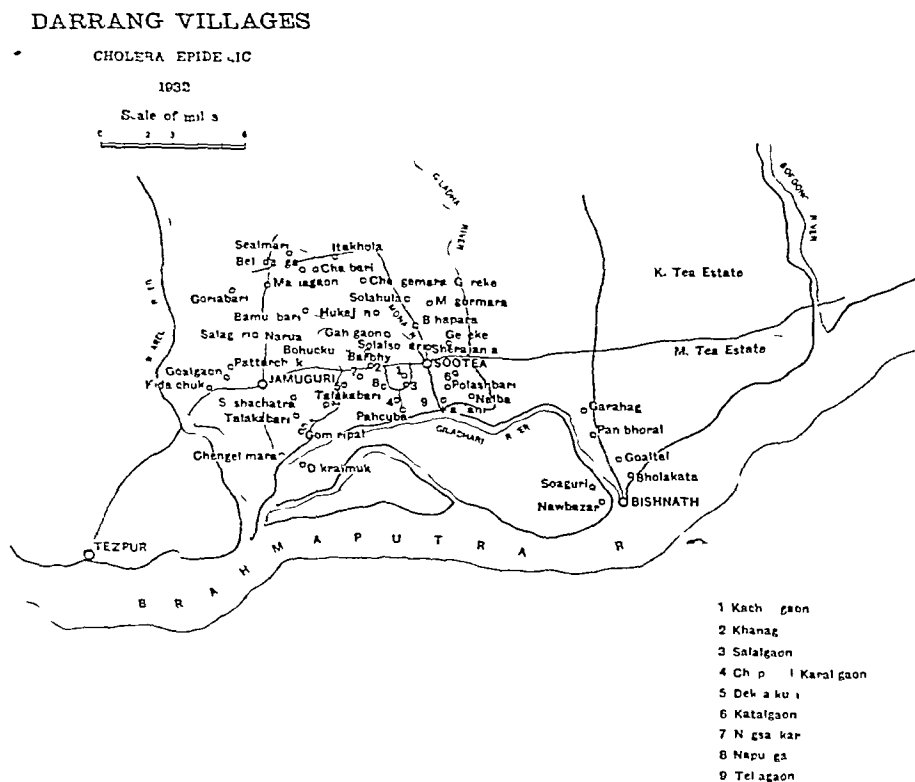
The epidemic in the villages of the Darrang district was spread over the wide area lying between Bishnath and the Bhareli river (Map 3). This area, which is near the Brahmaputra river, is low and sandy, extending back from the Brahmaputra for three or four miles before any rise is noticeable. Here lies the Gilidhary river, an old bed of the Brahmaputra. This dying river takes its origin from the Brahmaputra at Bishnath and empties again into the Brahmaputra at the mouth of the Bhareli river. Several small streams, the Dikrai, the Monai and the Little Gilidhary, drain into the Gilidhary. Between the Gilidhary and the Brahmaputra lies a large sandy bank deposited by the main river on which grows thatch grass gathered by the villagers for thatching their houses. The Gilidhary is also a fishing stream for the district during the cold weather months, large numbers of fishermen from Behar Province migrate here during that period.

The portion of the area inland from Jamaguri and Sootea is only slightly higher than the Brahmaputra river and is also of sandy soil until near Sealmai, Itakhola and Chengamaia Gereke where the soil is heavier and where the tea estates, infected later (group 'B'), are located.

Bishnath, at the beginning of the Gilidhary, is a small town near which fishermen from Behar camp yearly from October to March. The town of Bishnath itself consists of many Hindu castes with some Mohammedans. The population between Bishnath and the Bhareli river is also mixed for, besides the Assamese villagers, many ex-tea-estate labourers from gardens in Darrang have settled in this neighbourhood. Largely as a result of this settling, small villages have sprung up in which, in any one village, may be found people from various parts of India and of many castes. The villagers are dependent upon cultivation of rice land. Some take casual work on the nearby tea estates. The area of this epidemic is principally cultivated rice land with small villages and the hamlets scattered on the higher land. The Dikrai, Monai and Little Gilidhary wind through the district. Water supplies in the villages are from surface wells, few use other water unless a village is immediately on the banks of a stream.

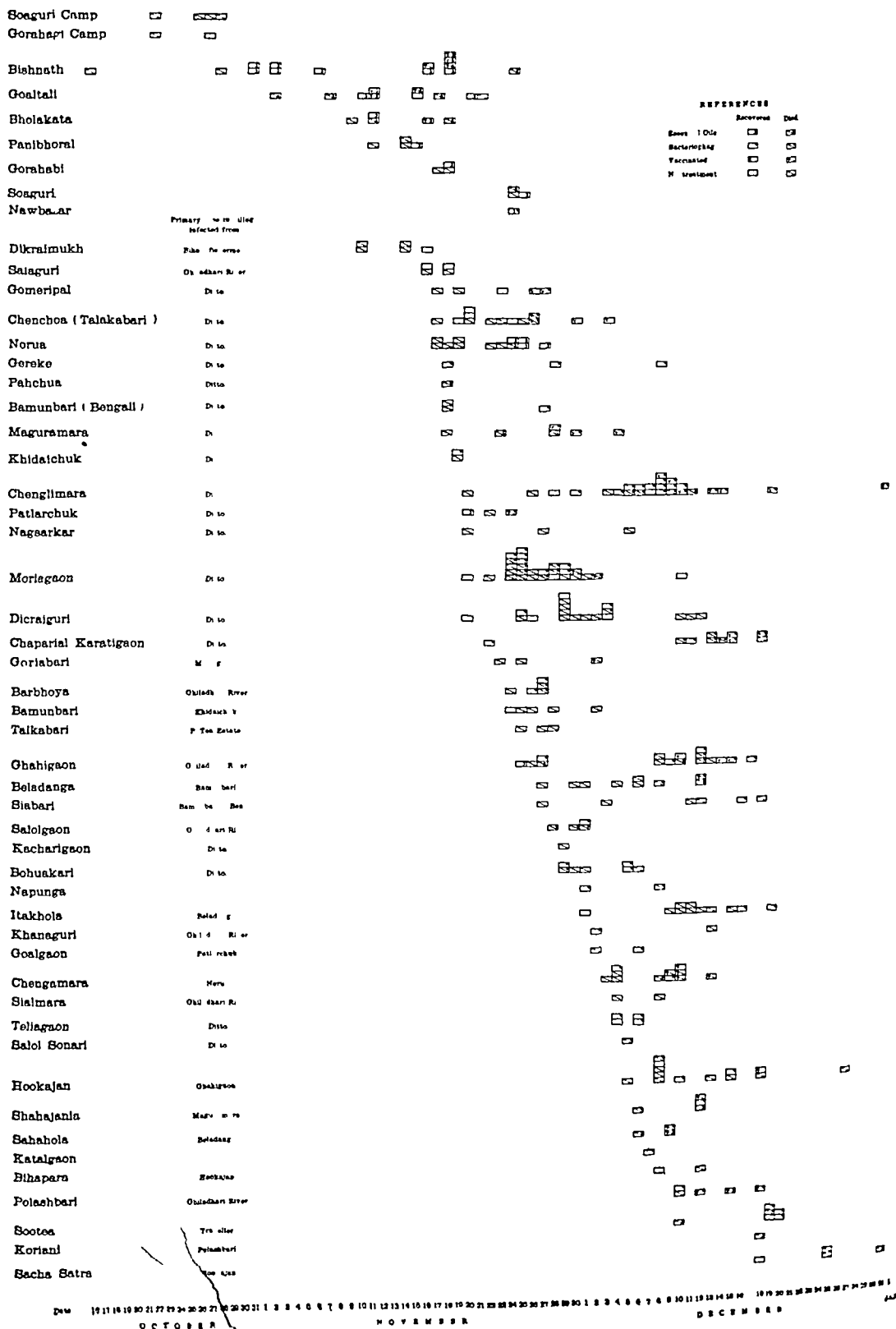
The first case of cholera appeared in the town of Bishnath on 16th October (Graph 7). This man had been to the bazaar at the M tea estate two days before when cholera was known to be present. He died on the 19th, having received no treatment. No further cases were traced to him. The next two cases were Behari fishermen from two separate camps who had gone together to the M tea estate bazaar on 20th October and developed cholera, each in his own camp, on the 22nd, both died on the same day. The one camp (Garahagi) of 63 men broke up as soon as the cholera case died. The individuals scattered to other fisher camps in the same

MAP 3



neighbourhood. At the second camp (Soaguri) three cases developed on the 26th, 27th and 28th, when the fishermen of this camp, numbering about 75, also scattered. Some men from both these camps went to Bajmani camp and others set up small camps of from three to 10 men elsewhere in the neighbourhood. Still others left for their homes in Behar, travelling via Silghat (Nowgong) and Amingaon (Kamrup). It was difficult to get information from these Behari fishermen as they feared that Government would stop the sale of their fish if it were known that they had cholera in their camps. During the time that cholera was present in these

DARRANG VILLAGES CHOLERA EPIDEMIC, 1932.



camps, fish was being sold by them in Bishnath and other bazaars. We obtained information of 18 cases in the fisher camps of whom 16 had died, others that recovered had left the camps.

Apart from the fishermen, 39 cases of cholera occurred in and near Bishnath between 28th October and 25th November, the date of the last-known case here.

When the fisher camps broke up, a few fishermen went further down stream to another camp on the Gildhary river between Gomiripal and Dikramukh. Subsequently, on 10th November, two cases occurred here among fishermen who had not been to camps near Bishnath, and this was followed by other cases on the 14th and 16th. At this time villagers from the whole area, except from the Bishnath group of villages, were busy collecting thatch from the land between the Gildhary and the Brahmaputra rivers. Batches of from 4 to 15 men, each from several villages collected here. These villagers camped along the banks of the Gildhary river and on the 17th and 18th cholera appeared among them in several places. There were three deaths here on the 18th and the body of one man who died then had been seen in the Gildhary river for three days before being swept away. On the 17th and 18th there was a stampede among these villagers who left their thatch cutting to return to their respective villages. Some had developed cholera and tried to struggle home, others developed it on the road home. Thus, the primary cases were spread over this large area within a few days. Later, men from other villages visited this area, became infected, and returned to their villages with cholera.

As has been stated, many of these villages had populations of mixed castes, ex-tea labourers, and, in the villages where this was so, cholera did not spread from house to house. On the other hand, there were a few villages occupied by peoples of the same caste and in these the disease spread rapidly. As instances of the latter, from Moriagaon, inhabited by Mohammedans, four men had gone to the Gildhary to fish and cut thatch. One returned to the village on 20th November with cholera, the other three, returning on the 21st, developed cholera on the 22nd, 24th and 25th. Subsequent to the return of these four men, 24 other cases occurred in this village with a population of between 150 to 200. The water-supply here was from a well near the house of the case returning on the 20th. Similarly, at Senchua (Talika-bari), a village of 20 Kaibarta houses with a population of approximately 100 and another 28 Assamese houses with a population of about 200, a Kaibarta returned from the Gildhary with cholera on 17th November. After his return, 12 other cases occurred in seven Kaibarta houses all taking their water from wells near the first case. No case occurred among other Assamese, living within about 40 yards, who took their water-supply from their own wells.

At Chenglmara, another Kaibarta village half a mile from the Gildhary, with about 100 houses and a population of 450, a man, who had cholera for two days while at a thatch cutting camp, returned to his home on 22nd November and died on the 27th. No other case occurred until 4th and 5th December when two men living close to the first case were attacked. Following on this 23 other cases developed among the Kaibarta villagers.

At Norua, a Koch village, five cases returned from the Gildhary and subsequently seven other cases developed.

garden managers, the people were not under the same risk as villagers. The labourers did not get their primary infection from the Gildhary area. In P, T and D estates the first case had visited an infected house in one of the villages. In the M estate the primary infection was from the Monai river, by which infection was brought from estate T. In estate S three primary cases all received their infections when fishing in a small stream.

P tea estate

This is a large estate with several sets of lines in different parts of the garden. Only one line was affected during the epidemic and this line was located by the side of the Little Gildhary river, which is used for bathing and washing clothes. Water-supply is from wells but some labourers doubtless used river water as well. The first two cases occurred on 27th November both having visited a known cholera case in a nearby village. These cases were treated in hospital and the next case to occur was that of the hospital sweeper on 2nd December. All of these cases died. On 13th December another case occurred, followed by 12 others between that date and the 27th. Of these cases 10 had been cutting a rice field near a known infected village and had taken water from there. The labourers on this estate were infected from an outside source and, having been treated in hospital, and preventive measures being put into force, there was no great spread of the disease.

T tea estate

This estate has several lines, two of which were affected by cholera. Lines 8 and 26 are both on the Monai river as were, also, two long buildings housing Mikir cold weather labourers. The first case of cholera occurred on 5th December in a man who had been visiting and nursing a cholera case in a nearby village. His wife developed cholera on the 8th and, between that date and the 20th, 14 other cases occurred all of whom took their water-supply from the Monai river not more than a quarter of a mile below the house of the first and second cases. Of the 16 cases six were in line 26, the two first cases were in one house, four were in separate houses within 100 yards and down stream from the originally infected house. One case occurred in each of the two houses occupied by Mikirs. In line 8, consisting of about 20 houses, only two adjacent houses were infected, one having six cases and the other two. The first two cases occurred on the 9th, the seven subsequent cases occurred between the 10th and the 22nd.

M tea estate

This estate is located $1\frac{1}{2}$ miles below the T estate on the Monai river and two days after the first case on the T estate the first case occurred here. There was no evidence that the child who was the first case on this estate had been away from her home except to play in the river and, knowing that the river was polluted farther up, it seems reasonable that this case was an extension from estate T. The Monai river water was used by the labourers housed in the central lines near the factory and, between 7th and 24th December, 18 cases occurred. Of these, two secondary cases occurred in the house of the first case, one other house had two

cases and the remaining 13 were all in separate houses. The distribution is that of a river infection.

S tea estate

This estate has several lines but only one line in the estate was infected. This line was occupied by labourers of the Munda caste, water being from a piped supply. The first case (7th November) gave no history of having been away from the garden. He had been fishing in the Sadharu stream with two persons, who also developed cholera on the 8th. For one of these cases that developed on the 8th a feast was held on the 9th and the balance of the eight cases which occurred after that date all attended this feast, other houses were thus infected.

D tea estate

This is a large estate having several lines but only one infected and that with only four cases. The first two cases on 16th December were in separate houses and are said to have had nothing in common. One, a man, had been out of the estate to an infected village where he had visited a cholera patient. The other, a child, had not been away but had been playing in the Monai river. The third case had visited a case of cholera in a nearby village while the fourth case was the husband of the third patient and had been nursing her during her illness. All were treated in hospital except the first case who died before she could be taken to hospital.

Part III

MORTALITY

SECTION I

Sibsagar epidemic

Of the 699 cases in the Sibsagar epidemic, we have complete data of 676. Three hundred and ninety-nine received no treatment and, of the balance (277), some were vaccinated before the onset of the disease, some received bacteriophage on different days after the onset and others received both vaccination and bacteriophage. The cases receiving bacteriophage after 48 hours are not in the same category as those receiving bacteriophage within the first 48 hours of the disease, for, in some cases irreparable damage had already been done and in others danger had already passed. We have therefore adopted the following classification of cases —

- I Cases receiving no treatment
- II Unvaccinated cases receiving bacteriophage *within* 48 hours of onset
- III Vaccinated cases receiving bacteriophage *within* 48 hours of onset
- IV Unvaccinated cases receiving bacteriophage *after* 48 hours of onset
- V Vaccinated cases receiving bacteriophage *after* 48 hours of onset
- VI Cases vaccinated before onset—'Vaccinated only'

Information as to the administration of vaccine or bacteriophage was obtained from the patient or his household and in nearly every case was verified by the evidence of more than one person.

The treatment in all village cases was in the hands of persons who were quite unaware of, and hence not interested in, the scrutiny to which the results of the treatment would be subjected. The diagnosis was that of the villagers themselves. There is probably less error in the diagnosis of epidemic cholera than in that of any other disease in India. Only one criterion of the results of treatment has been taken, recovery or death. It was immaterial whether the death occurred during the collapse stage or from uræmia, heart failure or an intercurrent infection later in the disease, all deaths are taken as deaths from cholera. This criterion—whether a person lived or died—applied by the members of the family and verified by one of us, was not less reliable than if applied by an expert medical man.

In one group in Sibsagar Town where the recovery rate was unusually high—the highest in the epidemic—the blood of ten of the recovered cases was tested for agglutination, together with ten of the laboratory staff, all of whom are daily working with cholera cultures. All of these ten Sibsagar sera agglutinated three different strains of cholera (Table V). Nine of the controls did not agglutinate and the tenth, vaccinated six weeks earlier, gave agglutination (Table VI).

TABLE

Agglutination reactions of sera of ten recovered cholera

(24 hr)

Patient's name.	STRAIN Sib—2077								S- Sib—				
	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{300}$	$\frac{1}{500}$	$\frac{1}{750}$	$\frac{1}{1000}$	Control	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{300}$	$\frac{1}{500}$
1 Saru Ram	++	++	+	-	+	±	-	-	++	++	+	+	=
2 Padum	++	+	±	-	-	-	-	-	++	+	±	-	-
3 Shoola	++	++	+	±	-	-	-	-	++	++	+	±	-
4 Niroda	++	++	+	+	-	-	-	-	++	++	+	+	-
5 Sarbeswari	+	-	-	-	-	-	-	-	+	-	-	-	-
6 Sarbeswar	++	++	++	++	++	+	+	-	++	++	++	++	-
7 Arti	++	+	±	-	-	-	-	-	++	+	±	±	=
8 Kusam	++	++	++	+	-	-	-	-	++	++	++	+	=
9 Rupaity	++	++	++	++	+	-	-	-	++	++	+	+	=
10 Silchale	++	++	+	+	-	-	-	-	++	++	+	+	-

V

cases with three strains of V cholerae

reading)

AIN 2029			STRAIN 1077								REMARKS
$\frac{1}{750}$	$\frac{1}{1000}$	Control	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{300}$	$\frac{1}{500}$	$\frac{1}{750}$	$\frac{1}{1000}$	Control	
-	-	-	++	++	+	+	±	-	-	-	Dilutions of sera were made with 0.8 per cent NaCl
-	-	-	-	-	-	-	-	-	-	-	
-	-	-	++	++	+	-	-	-	-	-	
-	-	-	+	-	-	-	-	-	-	-	
-	-	-	+	+	-	-	-	-	-	-	
+	-	-	++	++	++	++	++	++	+	-	
-	-	-	+	±	-	-	-	-	-	-	
-	-	-	++	+	+	±	±	±	±	-	
-	-	-	+	+	-	-	-	-	-	-	
-	-	-	++	++	++	+	+	+	±	-	

TABLE

Agglutination reactions of ten control sera from member

(24 hour

[illegible]

VI

of the staff with three strains of V cholerae

reading)

AIN 2029			STRAIN 1077								DATE OF CHOLERA VACCINATION
$\frac{1}{750}$	$\frac{1}{1000}$	Control	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{300}$	$\frac{1}{500}$	$\frac{1}{750}$	$\frac{1}{1000}$	Control	
-	-	-	-	-	-	-	-	-	-	-	Not vaccinated
-	-	-	-	-	-	-	-	-	-	-	" "
-	-	-	-	-	-	-	-	-	-	-	" "
-	-	-	-	-	-	-	-	-	-	-	" "
-	-	-	-	-	-	-	-	-	-	-	" "
=	TR	-	+	+	+	+	+	+	+	-	Vaccinated August 14, 1932
-	-	-	-	-	-	-	-	-	-	-	" June 1928
-	-	-	-	-	-	-	-	-	-	-	" July 1929
-	-	-	-	-	-	-	-	-	-	-	" July 1928
-	-	-	-	-	-	-	-	-	-	-	{ Cholera in 1880 Vaccinated 1916

Vaccinated cases we have taken as those who were inoculated with 1 c c of cholera vaccine containing 8 000 millions per c c or a lesser dose according to age, one or more days before they fell ill

Six cases vaccinated on the day they fell ill and a further five cases vaccinated during their illness or convalescence will be mentioned later

A case treated with bacteriophage we define as one who had received one or more ampules of bacteriophage at any time during the period of illness. Even if a case received bacteriophage when he was in a condition of collapse or when suppression of urine was established, he has been recorded as treated with bacteriophage. It was difficult to ascertain how many ampules of bacteriophage had been given to each case but so many asserted that only one had been given that we believe we are not wrong in stating that more than 95 per cent had only one ampule containing 2 c c

In a disease so fatal as cholera the evaluation of the use of bacteriophage on alternate cases in villages is not possible. It would certainly result in a riot should one form of treatment seem less efficacious than another. The alternate case method being impracticable, we have to compare the treated with the untreated and to apply every test we can to elicit differences in the groups that, apart from treatment, might affect the mortality. It is necessary, therefore, to make sure that all these populations are comparable in age, sex, caste and as regards the period in the epidemic in which the cases occurred. We have, therefore, in each instance, compared the observed distribution with what might be expected had the cases been distributed group by group in accordance with the proportion to the total. If groups show a significant deviation of the observed from the expected there would be evidence of selection. For example, if the numbers in any treated age group fell much below that expected, it would suggest that that age group is under-represented in the treated and over-represented in the untreated. Such groups cannot be compared.

(a) Age—

TABLE VII
Distribution of cases by age

Age groups	Untreated		Bacteriophage within 48 hours		Vaccinated, bacteriophage within 48 hours		Bacteriophage after 48 hours		Vaccinated, bacteriophage after 48 hours		Vaccinated only		Total
	O	E	O	E	O	E	O	E	O	E	O	E	
0-5	81	76.7	25	23.8	5	7.9	9	14.2	0	1.5	10	5.8	136
6-10	95	100.9	27	31.4	13	10.4	24	18.7	4	2.0	8	7.6	171
11-20	97	101.5	36	31.6	15	10.4	19	18.8	3	2.0	2	7.6	172
21-30	12	74.4	19	23.1	2	7.6	16	13.8	0	1.5	7	5.6	125
31-40	15	27.7	11	8.6	4	2.9	5	5.1	1	0.6	1	2.1	47
41-50	1	11.8	3	3.7	1	1.2	1	2.2	0	0.2	2	0.9	26
51-60	4	3.5	1	1.1	1	0.4	0	0.7	0	0.1	0	0.3	6
61-70	2	2.4	2	0.7	0	0.2	0	0.4	0	0.05	0	0.2	4
TOTALS	399		124		41		74		8		30		675

O — Observed

E — Expected

Table VII shows the observed and expected case distribution group by group for all age periods. The distribution of the expected cases fits closely that of the observed. There is no evidence of selection of cases for any treatment in any age group.

TABLE VIII
Mortality of untreated cases by age

Age groups	TOTALS		
	R	D	M
0-5	36	45	55.6
6-10	46	49	51.6
11-20	65	32	33.0
21-30	40	42	51.2
31-40	12	13	52.0
41-50	6	7	53.8
51-60	2	2	50.0
61-70	0	2	100.0
TOTALS	207	192	48.12

R —Recovered D —Died M —Mortality

Table VIII gives the mortality of the untreated cases in age groups. The lowest mortality of 33 per cent occurs in age group 11 to 20. The remaining groups, where the numbers are significant, have a mortality between 50 and 60 per cent. Except possibly in the one group, age had no effect upon the untreated mortality. Not only are we dealing with populations which are similar to each other in their age distribution, but the factor of age played no significant part in determining mortality in the untreated group. There is no evidence of increased resistance in older individuals. As we have already said, there had been no outbreak of cholera in this neighbourhood since 1917.

(b) Sex—

The percentage of females is 50.7

TABLE IX
Distribution of cases by sex

Sex groups	No treatment		Bacteriophage with in 48 hours		Vaccinated, bacteriophage with in 48 hours		Bacteriophage after 48 hours		Vaccinated, bacteriophage after 48 hours		Vaccinated only	
	O	E	O	E	O	E	O	E	O	E	O	E
Male	194	196.5	57	61.1	23	20.2	39	36.5	4	3.9	16	14.8
Female	205	202.5	67	62.9	18	20.8	35	37.5	4	4.1	14	15.2

O — Observed

 $\chi^2 = 1.90$ $n' = 6$

E — Expected

 $p = 0.85$

Table IX gives the observed and expected distribution for both sexes among the treated and untreated groups. The observed and expected distributions show a close approximation. There is no evidence of selection of cases by sex for any particular treatment. The populations are therefore reasonably similar in their sex composition.

TABLE X
Mortality of untreated cases by age and sex

Age groups	MALE			FEMALE			TOTALS		
	Recovered	Died	Mortality	Recovered	Died	Mortality	Recovered	Died	Mortality
0-5	22	21	48.8	14	24	63.2	36	45	55.6
6-10	20	27	57.4	26	22	45.8	46	49	51.6
11-20	29	16	35.6	36	16	30.8	65	32	33.0
21-30	15	11	73.3	25	31	55.4	40	42	51.2
31-40	5	11	68.7	7	2	28.6	12	13	52.0
41-50	6	6	50.0	0	1	100.0	6	7	53.8
51-60	2	1	33.3	0	1	100.0	2	2	50.0
61-70	0	2	100.0	0	0	0	0	2	100.0
TOTALS	99	95	48.97	108	97	47.32	207	192	48.12

Table X shows the mortality among the untreated male population which is 48.97 per cent, among females it is 47.32 per cent, with a mean of 48.12 per cent

As in the case of age, not only are the populations homogeneous as regards sex, but sex has had no influence on the untreated mortality, age group 31 to 40 is a possible exception to the statement that sex is without influence on mortality

(c) *Caste*—

TABLE XI

Distribution of cases by caste

Caste	No treatment		Bacterio phage with in 48 hours		Vaccinated, bacterio phage with in 48 hours		Bacterio phage after 48 hours		Vaccinated, bacterio phage after 48 hours		Vaccinated only	
	O	E	O	E	O	E	O	E	O	E	O	E
Mohammedan	31	39.5	15	12.3	5	4.1	8	7.3	1	0.8	7	3.0
Kalita	30	29.5	13	9.2	0	3.0	4	5.5	0	0.6	3	2.2
Ahom	58	59.6	15	18.5	3	6.1	20	11.1	2	1.2	3	4.5
Keot	18	14.8	2	4.6	2	1.5	2	2.7	0	0.3	1	1.1
Kaibarta	171	168.2	54	52.3	25	17.3	23	31.2	2	3.4	10	12.6
Koch	75	59.6	14	18.5	2	6.1	6	11.1	1	1.2	3	4.5
Others	16	27.7	11	8.6	4	2.9	11	5.1	2	0.6	3	2.1

O —Observed

E —Expected

Table XI shows that the distribution by caste of the untreated and treated, with the exception of the Kochs and the small group of 'other castes', is such as might be expected. No one caste received a particular treatment in preference to another.

TABLE XII

Mortality of untreated cases by caste

Caste	Recovered	Died	Mortality
Mohammedan	10	21	76.7
Kalita	18	12	40.0
Ahom	23	35	60.3
Keot	11	7	38.8
Kaibarta	92	79	46.2
Koch	44	31	40.2
Others	9	7	43.7
TOTALS	207	192	48.12

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Mohammedans and Ahoms have a mortality of over 60 per cent compared to a mean of 48.12 per cent

In Table XII $\chi^2 = 11.368$, $p = 0.08$, this is above the conventional line at 0.05 (Fisher, 1930) and seems to indicate that the variations in mortality due to caste are not significant

(d) *Period*—

TABLE XIII

Distribution of cases by period

Period	No treatment		Bacteriophage with- in 48 hours		Vaccinated, bacteriophage with in 48 hours		Bacteriophage after 48 hours		Vaccinated, bacteriophage after 48 hours		Vaccinated only	
	O	E	O	E	O	E	O	E	O	E	O	E
July 19 to 25	28	18.9	0	5.9	0	1.9	4	3.5	0	0.4	0	1.4
July 26 to Aug 1	74	59.6	12	18.5	0	6.1	15	11.1	0	1.2	0	4.5
Aug 2 to 8	150	151.7	53	47.1	17	15.6	31	28.1	2	3.0	4	11.4
Aug 9 to 15	104	109.8	34	34.1	14	11.3	16	20.4	5	2.2	13	8.3
Aug 16 to 22	30	38.4	15	11.9	7	3.9	5	7.1	0	0.8	8	2.9
Aug 23 to Sept 15	13	20.6	10	6.4	3	2.3	3	3.8	1	0.4	5	1.6

O — Observed

E — Expected

During the third and fourth periods the numbers in each group approximated closely the figures likely to be obtained in random samples. In the first and second periods the untreated exceeded, and in the last two periods were less than would have been expected by a proportional distribution. In other words, as the epidemic proceeded the chances of being treated were greater.

TABLE XIV

Mortality of untreated cases by period

PERIOD		Recovered	Died	Mortality
Number	Dates			
1	July 19 to 25	9	19	67 8
2	July 26 to Aug 1	29	45	60 8
3	Aug 2 to 8	79	71	47 3
4	Aug 9 to 15	62	42	40 4
5	Aug 16 to 22	22	8	26 7
6	Aug 23 to Sept 15	6	7	53 8
TOTALS		207	192	48 12

Table XIV gives the distribution of recoveries and deaths of each group according to the period in which the cases occurred. In this table there is a progressive

TABLE XV

Mortality of cases grouped according to period in which they occurred and the treatment they received

Sibsagar

Period	No treatment			Bacteriophage within 48 hours			Bacteriophage within 48 hours (vaccinated previously)			Bacteriophage after 48 hours			Bacteriophage after 48 hours (vaccinated previously)			Vaccination			Totals		
	II			III			IV			V			VI			VII			VIII		
I	R	D	M	R	D	ML	R	D	M	R	D	M	R	D	M	R	D	M	R	D	M
1 July 19 to July 25	9	19	67.8	0	0	0	0	0	0	3	1	25.0	0	0	0	0	0	0	12	20	62.5
2 July 26 to Aug 1	29	45	60.8	7	5	41.6	0	0	0	12	3	20.0	0	0	0	0	0	0	48	53	52.5
3 Aug 2 to Aug 8	79	71	47.3	42	11	20.7	14	3	17.6	23	8	25.8	2	0	0	2	2	50.0	162	95	37.0
4 Aug 9 to Aug 15	62	42	40.4	26	8	23.5	10	4	28.6	11	5	31.3	5	0	0	9	4	30.8	123	63	33.9
5 Aug 16 to Aug 22	22	8	26.7	13	2	13.3	5	2	28.6	4	1	20.0	0	0	0	6	2	25.0	50	15	23.1
6 Aug 23 to Sept 15	6	7	53.8	7	3	30.0	3	0	0	3	0	0	1	0	0	5	0	0	25	10	28.6
Totals	207	102	48.12	95	29	23.4	32	9	22.0	56	18	24.3	8	0	0	22	8	26.7	420	256	37.87

R—Recovered, D—Died, M—Mortality

reduction in mortality from 67·8 per cent in the first week to 26·7 per cent in the fifth week of the epidemic. Thirteen cases after 23rd August gave a mortality of 53·8 per cent. For the cases in the fifth and sixth periods, i.e., after 16th August, the mortality is 34·8 per cent.

Considering, therefore, the periods, the groups in Tables XIII and XIV are not random samples from similar populations. The cases early in the epidemic are not in the same class as those later in their chances of being treated. Nor, untreated, are they liable to the same risk of death.

The only fair comparison that can be made is between groups of cases occurring in the same period of time and in doing this, age, sex and caste can be neglected.

In Table XV each treatment group may be compared with the corresponding 'no treatment' group of the same period. When bacteriophage was given within 48 hours the reduction in the mortality requires no comment*. The figures (columns II and III) in the periods Nos 2 and 6 are small. Averaging periods 2 and 3, and 5 and 6, we have for three successive periods mortalities of 24·6, 23·5 and 20·0 per cent for the bacteriophage cases as compared to 51·8, 40·4 and 34·9 per cent in the untreated. The mortality of the bacteriophage treated cases drops at once to a nearly constant level. This is shown graphically in Graph 9. The same phenomenon is apparent in column IV. When we come to columns V and VI, i.e., cases treated with bacteriophage after 48 hours, we are dealing with a number who, many having got over the initial collapse, are on the way to recovery and the mortality, if low, cannot be ascribed to treatment. Once again the mortality is at a comparatively constant level.

The 30 vaccinated cases (column VII) show mortalities intermediate between the untreated and the bacteriophage groups both with and without vaccine†.

Cholera is in some cases so rapidly fatal that even when treatment is at hand every case has not the same chance of treatment. Some of the most severe cases will die within a few hours, before treatment can be given. In dividing cases into untreated and treated the untreated will, therefore, always have more deaths, for they include the severest cases. By taking alternate cases as they are admitted into hospital and distributing these equally between two forms

* A little comment is perhaps necessary. Grouping as the authors suggest into periods 2 and 3, 4, 5, and 6 and comparing bacteriophage treated within 48 hours with untreated gives χ^2 as follows —

Periods	χ^2
2 and 3	15·0
4	3·15
5 and 6	1·69

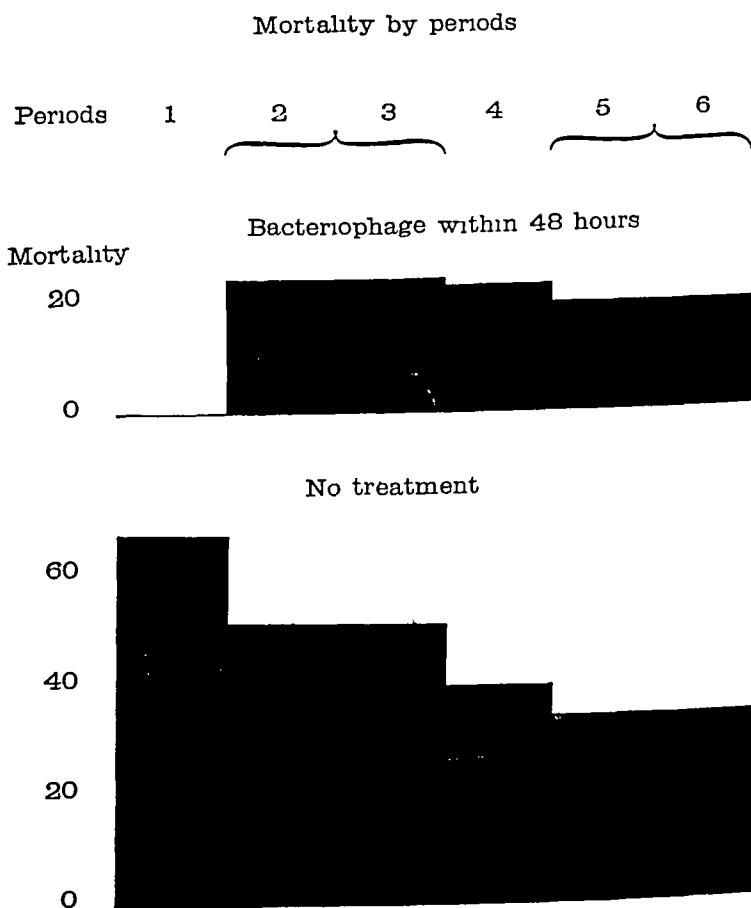
so that only the first period shows a definitely significant drop in mortality. Of course the fact that the mortality did decline (though not significantly) in the other two groups (in fact in all the groups examined) considerably strengthens the conclusion that the general drop was significant —(A B).

† The mortality among the 30 vaccinated cases does not differ significantly from that among the bacteriophage treated (even for all periods taken together when $\chi^2=0·24$). In the individual periods (2 and 3, 5 and 6) it is less but not significantly less than the untreated group. For all periods taken together it is significantly less ($\chi^2=5·16$) —(A B).

of treatment, both treatments are administered to selected populations, to populations in each of which a certain number of severe cases have already died. Any form of treatment will thus give a mortality below the real mortality of cholera, the mortality given by the total number of cases in an area and the total deaths.

GRAPH 9

SIBSAGAR CHOLERA EPIDEMIC 1932



In the grouping adopted, the untreated group is weighted with deaths that should have fallen into the treated group had it been possible to treat every alternate case from the first symptoms. On the other hand, the bacteriophage groups include every case to whom one dose of bacteriophage was administered even though the patient was *in articulo mortis*. A preliminary report published in the proceedings of the Conference of Research Workers at Calcutta (Indian Research Fund Association, 1932) gives a list of 13 cases which died before bacteriophage had time to

benefit them. These are all included in the groups receiving bacteriophage within 48 hours. They weight the mortality against bacteriophage and to some extent set off the weighted mortality of the treated cases.

The deaths occurring on the first day may, however, be excluded from both untreated and treated. This can readily be done for the untreated cases. For the treated cases, it is difficult. Treatment on the first day is more likely to begin towards the end of the first day than near the beginning. Indeed treatment on the 'first day' may include treatment within 30 hours of the onset if the patient takes ill during the evening. If we put down all cases treated on the first day as having got the treatment at the end of 24 hours then no deaths will be abstracted and the figures will certainly not be weighted in favour of bacteriophage.

TABLE XVI

Results	Untreated	Bacteriophage 'at the end of 24 hours'	Bacteriophage on the second day
Recoveries	207	53	42
Total deaths	192	15	14
Deaths within 24 hours of onset deducted	40		
Nett deaths	152	15	14
Mortality	42.3 per cent	22.05 per cent	25 per cent

It is noteworthy that when cases received bacteriophage on the second day and thus were in a population from which fulminating cases were definitely excluded, the mortality was not lower than for the cases treated on the first day in which group the fulminating cases fell. The effect of early administration of the bacteriophage seems to be significant.

The number of cases that received bacteriophage on the second day, i.e., between 24 and 48 hours after onset, was 56, of which 14 died, a mortality of 25 per cent.

We may assume that all these cases were of the type that would have survived the first 48 hours even without treatment, in the untreated group there were 288 cases of this type, of which 81 died, a mortality of 28.1 per cent. It is obvious therefore that the reduction in mortality effected by administration of bacteriophage on the second day is much less than that effected by administration on the first day. This is a very interesting result suggesting that we cannot expect any marked effect on mortality from the administration of bacteriophage on the second or subsequent days. The importance of having bacteriophage available in a village as against having it available in a hospital is thus obvious; this, we believe, accounts for the very low mortality in Nowgong as shown in Part I, where bacteriophage was available for early administration in the villages.

We have examined the Subsagar mortality in Table XLVI (see Appendix) for age and period, deducting the deaths that occurred on the first day and, in case this unloading might not be enough, the deaths that occurred on the first and second days. Of the 192 deaths in the untreated group, 40 died on the first day and 73 on the second day of illness, a total of 113. These have been subtracted from the untreated groups. Of the 29 deaths in the group of cases that received bacteriophage within 48 hours, 8 cases died on the first, and 12 died on the second day. These have not been deducted. The following is the result —

TABLE XVII
Subsagar mortality

	July 19 to 25		July 26 to Aug 1		Aug 2 to 8		Aug 9 to 15		Aug 16 to 22		Aug 23 on		Tot
	R	D	R	D	R	D	R	D	R	D	R	D	
Deducting deaths on 1st day	9	14	29	35	79	60	62	31	28	13	0	0	207
	60.4 per cent		54.7 per cent		43.2 per cent		33.3 per cent		31.7 per cent				42.5 per cent
Deducting deaths on 1st and 2nd days	9	8	29	16	79	32	62	14	28	8	0	0	207
	47.0 per cent		35.6 per cent		28.8 per cent		18.4 per cent		22.2 per cent				27.5 per cent
Bacteriophage within 48 hours as per table	7	5	42	12	26	7	13	2	7	3	0	0	23
	25.7 per cent				21.2 per cent		20.0 per cent						

The loading of the treated deaths by deaths on the first and second days may, however, be estimated by noting the deviation in both groups from the deaths that might be expected if these had been distributed proportionally

TABLE XVIII
Sibsagar mortality

		Died on first day	Died on second day	Died later	Recovered	TOTAL
Untreated	Observed	40	73	79	207	390
	Expected	36.6	64.85	67.14	230.4	
Bacteriophage within 48 hours	Observed	8	12	9	95	124
	Expected	11.4	20.15	20.86	71.6	
Difference		3.4	8.15	11.86	23.4	
χ^2 untreated		0.3158	1.0242	2.0950	2.3765	
χ^2 bacteriophage within 48 hours		1.0140	3.2964	6.7430	7.6474	

In abstracting 40 deaths from the untreated group instead of 3.4, much more has been done than is required

In villages dotted along the course of the Dikhu river (Map 2), which is not only the common sewer but also the common drinking supply, and where the infection has passed from above downwards we might also expect, as Harvey (1930) has suggested, that the villages lower down would receive bacteriophage excreted by the cases higher up and that, if the administration of bacteriophage in treatment lowers the mortality, the mortality would also be lowered along the lower reaches of the river. In Table XIX the observed and expected deaths among the untreated cases are shown for each village along the river bank from above downward. All the 10 villages on the immediate banks of the Dikhu river above and including Kariagaon, with one exception (Desial Meteka), have deaths in excess of the number expected amongst those receiving no treatment. Of the eight villages below Kariagaon which are on the immediate banks of the river, six of the eight have fewer deaths than might be expected. The two exceptions are Pathialal and Gatonga. The houses of the former village are located partially on the Dikhu

and partially on its subsidiary stream, the Namdang, extending to and connecting with Namdang

TABLE XIX

Villages observed and expected deaths arranged according to position on the Dikhu river

Villages	DEATHS		Observed to expected
	Observed	Expected	
Hansara	3	1 44	+
* Julagaon	0	0 48	—
Noali Christiangaon	2	0 92	+
Sibsagar	6	4 81	+
Arjunguri	1	0 48	+
Desial Meteka	5	6 74	—
Mathadang	15	12 03	+
Kamarphodia	9	7 22	+
Senchuagaon	4	2 41	+
Chaulkara	13	8 18	+
Kariagaon	6	4 33	+
Balaghat	15	16 84	—
Pathialal	18	16 84	+
Fakum	10	13 95	—
* Pathialal Namdang	6	6 26	—
* Namdang Kumargaon	2	1 93	+
* Phukenphodia	15	13 95	+
* Namdang Bangaligaon	14	14 44	+

* Villages not on immediate banks of the Dikhu river but where water is taken from streams emptying into the Dikhu (see Map 2)

TABLE XIX—*concl'd*

Villages	DEATHS		Observed to expected
	Observed	Expected	
Desial Jagichul	10	11.92	—
Kharidhara	9	10.11	—
Gatonga	3	1.93	+
Bharalungaon	8	12.51	—
Goalgvangaon	13	16.36	—
*Morisuti	5	2.89	+

* Near Desang river

It is noteworthy (*see* Graph 5) that, in the villages below Fikun which are on the banks of the Dikhu, and in a position to receive bacteriophage by the river from villages higher up, the epidemic tended to come to an end sooner. There were fewer straggling late cases. The epidemic had no tail. An absence of the tail is noticeable higher up the river in Sibsagar, Arjunguri, Desial and Mathadang where bacteriophage was given to most of the cases.

SECTION II

Darrang villages

As shown in Part II, the Darrang villages epidemic differed from the Sibsagar epidemic in that the water-supply in villages in the former was principally from wells, whereas, in the latter, all the villages took water either directly from the Dikhu river itself or from one of its tributaries.

Apart from the few villages infected in the immediate neighbourhood of Bishnath, most of the villages received their first infection from the Gildhary thatch cutting area (*see* Graph 7). Of the population who visited this area, all were men or boys old enough to assist in the cutting and carrying of thatch. Younger children, girls and women did not go but were infected later, hence there were more male than female cases.

We have data of 308 cases in this epidemic. Essential oils were used in 38 cases, 10 of whom had been previously vaccinated. Fifteen had been vaccinated previously but received no treatment after infection. Of the balance, 138 had no treatment at all, 83 received bacteriophage alone and 24 who had been previously vaccinated received bacteriophage.

We have adopted the same treatment groups as in the study of the Sibsagar epidemic data, except that we have included here the essential oil groups. Data

were collected in the same way as in Sibsagar and our definitions of vaccination and bacteriophage treatment are the same

TABLE XX
Distribution of cases by age
Daring villages

Age groups	No treatment		Bacteriophage with in 48 hours		Vaccinated, bacteriophage with in 48 hours		Bacteriophage after 48 hours		Essential oils		Vaccinated, essential oils		Vaccinated	
	O	E	O	E	O	E	O	E	O	E	O	E	O	E
0-5	13	11.6	5	5.7	2	2.0	1	2.2	1	2.4	1	0.8	3	1.3
6-10	16	13.9	3	6.7	3	2.4	3	2.6	1	2.8	2	1.0	3	1.5
11-20	25	24.6	11	12.0	7	4.3	5	4.6	4	5.0	2	1.8	1	2.1
21-30	27	34.5	24	16.8	0	6.0	9	6.5	10	7.0	3	2.5	4	3.1
31-40	28	28.2	13	13.7	8	4.9	5	5.3	6	5.7	1	2.0	2	3.1
41-50	15	15.7	8	7.6	4	2.7	3	3.0	4	3.2	0	1.1	1	1.1
51-60	11	8.1	3	3.9	0	1.4	0	1.5	2	1.6	1	0.6	1	0.6
61-70	3	1.3	0	0.7	0	0.2	0	0.3	0	0.3	0	0.1	0	0.1

O — Observed

E — Expected

Table XX shows the distribution of observed and of expected cases group by group for all the age periods. The proportion of younger children infected is not as high as in Sibsagar. There is no evidence of selection of cases in the treated group as compared to the untreated group, although the fit is not quite as close as is that of Sibsagar.

TABLE XXI
Mortality of untreated cases by age

Age groups	Recovered	Died	Mortality
0-5	3	10	76.9
6-10	6	10	62.5
11-20	4	21	84.0
21-30	5	22	81.5
31-40	1	27	96.4
41-50	3	12	80.0
51-60	1	10	90.9
61-70	0	3	100.0
TOTALS	23	115	83.3

Table XXI gives the mortality of the untreated cases in age groups. The lowest mortality figures are for children aged 10 and under. Above the age of 10, the mortalities are higher. The possible reason for this is that children of these ages were infected in their own houses and did not travel while ill, but the groups may be too small to be significant. The mortality for the ages 31 to 40 is 13 per cent higher than the average and for those over 50 years of age the mortality is over 90 per cent. There is no increased immunity in any age group. We have no evidence of immunity arising from previous epidemics. For such small populations the figures seem remarkably uniform.

Sex

Of the total of 308 cases, 180, or 58.4 per cent, were males. The reason for the predominance of males has been explained.

TABLE XXII

Distribution of cases by sex

Sex groups	No treat ment		Bacterio phage with in 48 hours		Vaccinated bacterio phage within 48 hours		Bacterio phage after 48 hours		Essential oils		Vacci rated, essential oils		Vacci nated		Totals
	1		2		3		4		5		6		7		
	O	E	O	E	O	E	O	E	O	F	O	F	O	E	
Male	90	80 6	36	39 2	11	14 0	17	15 2	17	16 4	3	5 8	6	8 8	180
Female	48	57 4	31	27 8	13	10 0	9	10 8	11	11 6	7	4 2	9	6 2	128
TOTALS	138		67		24		26		28		10		15		308

O—Observed

E—Expected

$\chi = 10.148$ $n' = 7$ $p = 0.12$

Table XXII gives the observed and expected distribution for both sexes among the treated and the no treatment groups. Proportionately fewer males were treated.

than females for all forms of treatment, but $\chi^2 = 10.148$ and $p = 0.12$. This distribution might be expected 12 times out of 100.

TABLE XXIII

Mortality of untreated cases according to age and sex

Age groups	MALE			FEMALE			TOTALS		
	Recovered	Died	Mortality	Recovered	Died	Mortality	Recovered	Died	Mortality
0-5	1	6	85	2	4	66	3	10	76.9
6-10	4	6	60	2	4	66	6	10	62.5
11-20	2	13	82	2	8	80	4	21	84.0
21-30	5	13	70	0	9	100	5	22	81.5
31-40	1	20	95	0	7	100	1	27	96.4
41-50	1	7	85	2	5	71	3	12	80.0
51-60	1	8	80	0	2	100	1	10	90.9
61-70	0	2	100	0	1	100	0	3	100.0
TOTALS	15	75	83.3	8	40	83.3	23	115	83.3

Table XXIII shows that males and females receiving no treatment had each a mortality of 83.3 per cent.

Caste

As 62 castes were represented in the 308 cases, the numbers for any individual caste are too small to give significant results.

TABLE XXIV

Distribution of cases by period and treatment

Period	No treat- ment		Bacterio phage with in 48 hours		Vaccinated, bacterio phage within 48 hours		Bacterio phage after 48 hours		Essential oils		Vaccinated, essential oils		Vaccinated		Totals
	O	E	O	E	O	E	O	E	O	E	O	E	O	E	
Oct 16 to Nov 11	5	81	0	39	0	14	0	15	10	16	3	06	0	09	18
Nov 12 to 18	16	134	0	65	0	23	2	25	6	27	5	10	1	15	30
Nov 19 to 25	37	260	1	126	1	45	11	49	7	527	1	19	0	28	58
Nov 26 to Dec 2	29	282	18	137	4	49	3	53	4	57	0	20	5	31	63
Dec 3 to 9	24	282	28	137	5	49	5	53	0	57	0	20	1	31	63
Dec 10 to 16	19	246	13	120	11	43	4	46	1	50	0	18	7	27	55
Dec 17 to Jan 6	8	94	7	46	3	16	1	18	0	19	1	07	1	10	21
TOTALS	138		67		24		26		28		10		15		308

O—Observed

E—Expected

In Table XXIV for the untreated (column II), the distribution of cases period by period is close to a proportional distribution. Among the different treatment groups, all, with the exception of the essential oil groups, show that bacteriophage and vaccine did not come into the picture to any extent until the third and fourth periods. That the number of untreated were as close to a chance distribution at the beginning as at the end of the epidemic was due to the essential oils being used in the early part of the epidemic near Bishnath and later, when the epidemic extended to Sootea-Jamaguri area, this treatment was replaced by bacteriophage.

TABLE XXV

Mortality of untreated cases by period

Period	Recovered	Died	Mortality
Oct 16 to Nov 11	0	5	100
Nov 12 to 18	2	14	87.5
Nov 19 to 25	9	28	75.7
Nov 26 to Dec 2	4	25	86.2
Dec 3 to 9	5	19	79.2
Dec 10 to 16	1	18	94.7
Dec 17 to Jan 6	2	6	75.0

$$\chi^2 = 5.256, n = 7, p = 0.513$$

In Table XXV there is no evidence of reduction in the mortality period by period. The mortality is remarkably uniform and the value of ' p ' shows that this distribution of recoveries and deaths might be expected to deviate, more than that observed, by chance, 50 times out of 100.

In the Dairang villages the infection came from the Gilidhary river and was distributed to a number of different centres in each of which there was a small community too small for the epidemic to carry on. The villages that were secondarily infected from these small outbreaks were largely infected from the first cases, i.e., before natural bacteriophage from a recovering patient was likely to be present. As each small community had its own well, there was little opportunity for bacteriophage to be distributed by convalescent cases. If the progressive diminution of the mortality in Sibsagar is ascribable to the natural distribution of bacteriophage by the Dikhu river, we have in the Dairang villages epidemic an example of a continuous high mortality throughout an epidemic where conditions for the dissemination of bacteriophage are not present.

TABLE XXVI

Mortality of cases grouped according to treatment received and the week of the epidemic when they occurred

Darrang villages

Period	No treatment			Bacteriophage within 18 hours			Bacteriophage within 18 hours vaccinated previously			Bacteriophage after 18 hours			Bacteriophage after 18 hours vaccinated previously			Essential oils			Essential oils vaccinated previously			Vaccinated previously			Totals		
	R	D	M	R	D	M	R	D	M	R	D	M	R	D	M	R	D	M	R	D	M	R	D	M	R	D	M
Oct 16 to Nov 11	0	5	100													7	3	30	3						10	8	114
Nov 12 to 18	2	14	88							1	1	50				4	2	33	3	2	10	1	100		10	20	607
Nov 19 to 25	9	28	76	1	0	0				0	4	10				4	3	43	1						22	30	621
Nov 26 to Dec 2	4	25	86							2	0	0				2	2	50							23	10	631
Dec 3 to 9	5	19	79							3	2	50	1									1	100		28	35	555
Dec 10 to 16	1	18	95							8	3	27				1						3	4	57	21	34	618
Dec 17 to Jan 6	2	6	75	3	4	57				0	1	100							1			1			10	11	524
Totals	23	115	833	37	30	145	17	7	292	13	10	135	1	2	667	18	10	357	8	2	200	7	8	533	124	184	597

Comparing now the cases in the Darrang villages epidemic that had received bacteriophage within 48 hours with those untreated, the mortality in the former varies from 44 to 57 per cent in the groups in which the mortality in the untreated cases varied from 75 to 94·7 per cent (Table XXVI). The average mortality of the bacteriophage cases was 44·5 per cent as against 83·3 per cent for the untreated cases. In a more virulent epidemic than that of Sibsagar, the mortality is reduced to nearly half. As mentioned, when considering the Sibsagar epidemic, some cases were so rapidly fatal that even when treatment was at hand, every case had not the same chance of treatment. We attempted in an evaluation of the results of bacteriophage treatment to take this into account. This we did by deducting the deaths occurring within 24 hours in the no-treatment group in order to liken them to that of the bacteriophage group, the members of which could not have received treatment for some hours after the onset of the disease.

In Table XXVII we have treated the Darrang villages groups in the same way.

TABLE XXVII

Results	Untreated	Bacteriophage 'at the end of 24 hours'	Bacteriophage on the second day
Recoveries	23	20	17
Total deaths	115	16	14
Less deaths within 24 hours of onset	33		
Nett deaths	82	16	14
Mortality	78·1	44·4	45·2

After deducting, as we did in the Sibsagar figures, the fulminating cases from the untreated group and leaving the bacteriophage group untouched the mortalities are 78·1 per cent for the untreated, 44·4 per cent for those receiving bacteriophage in 24 hours and 45·2 per cent for those that received bacteriophage after 24 hours.

The mortalities in the remaining groups are given in Table XXVI. The numbers of cases in each group varied from 10 to 28, numbers too small to have weight, but, such as they are, the figures suggest that essential oils, a widely accepted treatment in India, is not without value in reducing mortality.

SECTION III

Darrang tea estates, groups 'A' and 'B'

In these tea estate epidemics bacteriophage was not used. They illustrate what happens when early removal to hospital and treatment by hypertonic saline

and permanganate are possible. By isolation, disinfection and vaccination the managers made every effort to stop the spread of the disease. The estate epidemics were moreover part and parcel of the Darrang village epidemics, so much so that, had other conditions been similar, we might fairly expect the mortalities to have been alike.

TABLE XXVIII

Treatment	GROUP 'A'			GROUP 'B'			TOTAL		
	Recovered	Died	Mortality	Recovered	Died	Mortality	Recovered	Died	Mortality
No treatment	0	10	100	0	11	100	0	21	100
Hypertonic saline and permanganate	20	12	37.5	13	17	56.6	33	29	46.8
Vaccinated, hypertonic saline and permanganate	15	6	28.6	9	8	47.1	24	14	36.8
Permanganate	11	3	21.4	0	0	0	11	3	21.4
Vaccinated previously	0	6	100.0	0	7	100.0	0	13	100.0
TOTALS	46	37	44.6	22	43	66.2	68	80	54.1

Table XXVIII shows the results of treatment. Every case that could be removed to hospital before death received treatment hence the mortality among the untreated and the vaccinated is 100 per cent. Five of these 34 died on the second day of illness, the remaining 29 died on the day they took ill. The cases receiving permanganate only were milder cases not requiring transfusion.

Grouping the unvaccinated cases receiving hypertonic saline and permanganate with those receiving permanganate alone, i.e., the severe and the mild cases, the mortality for hospital cases is 42 per cent as compared with 44.5 per cent in the villages where cases received one ampule of bacteriophage and no hospital attention.

Part IV.

INFECTIVITY

(In collaboration with B N Rau, Esq, CIE, ICS)

Sibsagar Town is on the banks of the Dikhu, but, unlike the villages also attacked in this epidemic, the town, as we have mentioned in Part I, has an excellent water-supply used by all except a small community of fishermen (Kaibartas) living in about 90 houses in that part of the town which is on the actual bank of the river. Of this community a child who played on the banks of the river, took ill on 26th July. The child died on 29th July. On the 29th an 'Aimata' ceremony was held to intercede for the life of the child (Ai, means The Great Mother, Mata, Calling). At this ceremony the children of that fisher community were fed by the parents of the sick child at their house. Within six days 23 children with two of the mothers were attacked with cholera. No person who had not attended the feast was affected during the period. The 25 cases were distributed among 17 houses. From these 25 cases there followed in the same and in adjoining houses 49 more cases. Some of the initial cases arising from the feast received bacteriophage others did not.

TABLE XXIX

Houses where bacteriophage was not given to the first case

Case	Number of initial cases	Number of people in house	Number of second wave cases in house	REMARKS
13	1	7	3	
17	1	7	0	
31	1	5	1	
33 and 34	2	3	0	
35*	1	7	1	* This case died in 11 hours, bacteriophage was given just before the death
40†	1	7	1	† This case died on the first day and had bacteriophage just before death
62	1	5	0	
7 houses	8	41	6	

TABLE XXIX—*concl'd*

Houses where bacteriophage was given to the first case

Case	Number of initial cases	Number of people in house	Number of second wave cases in house	REMARKS
6 and 7	2	8	0	
19	1	7	0	
20, 21 and 22	3	7	0	
23 and 24	2	7	0	
25	1	3	0	
26* and 27	2	9	0	* Died on first day before bacteriophage could be given No 27 received bacteriophage later that day and recovered
37	1	7	1	
43 and 44	2	10	0	
49	1	12	0	
51† and 55	2	5	1	† Died in six hours before bacteriophage could be given No 55 received bacteriophage and recovered
10 houses	17	74	2	

Table XXIX shows the distribution of the subsequent cases in the same houses. The numbers, though small, seem to indicate that fewer cases followed when the initial cases were treated with bacteriophage and more cases when they were not so treated, for, with twice the number of initial cases and nearly twice the population at risk, the subsequent cases in the treated group were one-third of those in the untreated groups.

Putting the data from the Sibsagar and the Darrang villages (Tables LIV and LV—see Appendix) in fourfold tables (Table XXX) we find that, where the primary cases were treated with bacteriophage the escapes were more and the secondary cases were significantly less than the expected

TABLE XXX

Sibsagar

	OBSERVED		EXPECTED		
	Bacteriophage within 48 hours to primary case	No treatment	Bacteriophage within 48 hours to primary case	No treatment	Total population at risk
Escapes	357	1,513	333 509	1536 491	1,870
Attacks	12	187	35 491	168 509	199
TOTALS	369	1,700	369	1,700	2,069

$$\chi^2 = 20.838, \quad n = 1, \quad p = \text{less than } 0.001$$

Darrang

	OBSERVED		EXPECTED		
Escapes	165	358	154 83	368 17	522.5
Attacks	7	51	17 17	40 83	58
TOTALS	172	409	172	409	581

$$\chi^2 = 9.506, \quad n = 1, \quad p = \text{less than } 0.002$$

We have seen that the treated and untreated populations in the village epidemics from which these are taken were reasonably homogeneous

To test this further, we have, in Tables LIV to LVII (*see* Appendix), divided the treated and untreated cases, taken in weekly periods, into houses with similar populations and with similar numbers of cases occurring on the first day. If the figures in the column of totals for the untreated cases are compared with those treated within 48 hours with bacteriophage, both groups having the same house populations, we find in every instance the subsequent or 'secondary' cases to be relatively fewer in the treated group. Again, taking the totals at the foot of the weekly periods we find the treated groups to have relatively fewer secondary cases in every instance. A scrutiny of the figures as they are is probably itself sufficient to give weight to the view that bacteriophage, if given to the first case in a house, does reduce the number of subsequent cases.

If we wish to test the significance of this reduction, we have to estimate how many cases should have occurred in the treated groups had the number of subsequent cases been proportionate to those of the untreated group.

Under different conditions the distribution of the secondary cases may be proportionate to the primary cases, to the population at risk, to the arithmetic or to the geometric mean of these two numbers. We need not in this paper discuss reasons that may be put forward for the selection of one or other of these factors but content ourselves with the factor which gives an 'expected' distribution of the untreated cases in closest agreement with the 'observed'. For this we have selected the arithmetic mean of the primary cases and the persons at risk in the same houses. This gives the closest approximation to the untreated cases of any factor we have taken.

The Sibsagar and Darrang epidemics, as we have seen, differed in their environment, in their mode of spread and in mortality. They are two independent tests. In both epidemics we have seen that the treated and untreated populations were remarkably homogeneous in age groups, in sex and in caste.

To secure that the two factors, (*a*) primary cases, and (*b*) persons at risk, are similar for the untreated and for the treated, we have taken houses with one primary case and grouped these according to the number of people in the house. In Sibsagar the households in which the first case was treated with bacteriophage numbered from 2 to 14 persons in a house (Table LIV—*see* Appendix). We have, therefore, taken the corresponding house groups from the untreated. Similarly for Darrang (Table LV—*see* Appendix), the house groups occurring in both untreated and treated houses had populations 2, 3, 8 and 11 in a house. As far as possible every known condition is comparable between the treated and untreated houses. In Sibsagar the 153 secondary cases in the untreated houses have been distributed according to the value of $\frac{a+b}{2}$. The expected cases for each house population group may be calculated by taking the ratio of the attacked (*y*) or in the association table (*m*), to half the total population and multiplying half the population in each house group by this ratio. For the treated houses the expected cases are calculated by the same ratio, the assumption being that the infectivity in the treated is the same as in the untreated houses.

TABLE XXXI
Sibsagar
Untreated

Persons in house	<i>a</i>	<i>b</i>	$\frac{a+b}{2}$	<i>m</i>	<i>m</i> ₁	<i>m</i> ₁ — <i>m</i>	(<i>m</i> ₁ — <i>m</i>)	$\frac{(m_1-m)^2}{m_1}$
2 and 3	19	29	24	2	4 835	—2 835	8 0372	1 6623
4	21	63	42	6	8 461	—2 461	6 0565	9 7158
5	35	140	87 5	14	17 627	—3 627	13 1551	0 7463
6	44	220	132	35	26 591	8 409	70 7113	2 6592
7	42	252	147	33	29 613	3 387	11 4718	0 3874
8	15	105	60	16	12 087	3 913	15 3116	1 2668
10	17	153	85	15	17 123	—2 123	4 5071	0 2632
11	8	80	44	10	8 864	1 136	1 2905	0 1456
12	12	132	72	7	14 504	—7 504	56 3100	3 8824
13 and 14	10	122	66	15	13 296	1 704	2 9036	0 2184
TOTALS	223	1 296	759 5	153	153 001			$\chi^2 = 11 9474$

Factor — $\frac{153}{759\ 5} = 0\ 20145,$ $n' = 10$ $p = 0\ 216$

Treated

2	1	1						
3	6	12						
4	3	9	33 5	1	6 7485	—5 7485	33 0453	4 8967
5	7	28						
6	9	45	27	0	5 4391	—5 4391	29 5838	5 4391
7	14	84	49	4	9 8710	—5 8710	34 4686	3 4919
8	7	49	28	3	5 6406	—2 6406	6 9728	1 2362
10	4	36						
11	1	10						
12	3	33	77	4	15 5115	—11 5115	132 5115	8 5430
13	3	36						
14	2	26						
TOTALS	60	369	214 5	12	43 2107			$\chi^2 = 23\ 6069$

Factor —Same as above, $n'=6,$ $p=0\ 0003$

The secondary cases in the houses where bacteriophage was given within 48 hours to the primary cases were 12 instead of the expected 43 2, a reduction of 72 per cent. The value of 'p' for the untreated houses is 0 216. This is well above the conventional line drawn by Fisher at 0 05 and indicates that there is no reason to suspect the hypothesis that the secondary cases are distributed according to the value of $\frac{a+b}{5}$. For the treated houses $p = 0\ 0003$, a value well below 0 05 and

indicates that something has come in to upset the hypothesis as applied to untreated cases

TABIE XXXII

Darrang villages

Untreated

Persons in house	<i>a</i>	<i>b</i>	$\frac{a+b}{2}$	<i>m</i>	<i>m</i> ₁	<i>m</i> ₁ - <i>n</i>	(<i>m</i> ₁ - <i>m</i>)	$\frac{(m_1-m)}{m_1}$
2	10	10	32.5	3	5 1223	2 1223	4 5042	0 8793
3	15	30		8	5 9891	2 0109	4 0137	0 6752
4	19	57		1	2 3641	1 3641	1 8698	0 7871
5	6	24	15	9	6 1467	2 8533	8 1413	1 3245
6	13	65	39	1	2 2065	-1 2065	1 4556	0 6598
7	4	24	14	7	7 1712	0 1712	0 0293	0 0041
8	10	70	45.5					
11	1	10						
TOTALS	78	290	184	29	28 9999			$\chi^2=4\ 3300$

$$\text{Factor} = \frac{29}{184} = 0\ 15761, \quad n' = 6 \quad p = 0\ 505$$

Treated

2	3	3	53	1	8 3533	-7 3533	54 0710	6 4731
3	8	16						
4	9	27						
5	8	32	40.5	4	6 3832	-2 3832	5 0796	0 8898
6	5	25						
7	3	18						
8	1	7						
11	2	20						
TOTALS	39	148	93.5	5	14 7365			$\chi^2=7\ 3629$

$$\text{Factor} = \text{Same as above} \quad n' = 3, \quad p = 0\ 026$$

In the Darrang villages, the secondary cases in the houses where bacteriophage was administered to the primary cases within 48 hours were five and the expected 14.7, a reduction of 66 per cent. In the untreated group the value of *p* is 0.505, showing that, as in Sibsagar, the fit of the expected cases to those observed is significant. In the treated group *p* is 0.026, again indicating a significant divergence. In both tables the fact that every observed value is less than the expected in the treated groups strengthens the conclusion.

TABLE XXXIII

Showing the values of C Index of infectivity in different untreated and treated groups in four epidemics

Treatment given to primary case or cases	No treatment		Bacteriophage within 48 hours		Bacteriophage within 48 hours, vaccinated previously		Bacteriophage after 48 hours, vaccinated previously			Vaccinated only	Essential oils	Permanganate	Permanganate and hypertonic saline	Permanganate and hypertonic saline, vaccinated previously
	1	2	1	2	1	2	1	2	3	1	1			
Number of primary cases per house														
Subangar	192	222	56	(111)	39	(0)	149	(95)	(0)	(51)	0			
Darrang villages	203	(270)	66	(250)	62	(0)	119	(0)			(0)	102		
Darrang tea estate, group 'A'	(320)	(333)									(148)*	95*	46*	33*
Darrang tea estate, group 'B'	(98)	(333)									(210)*		33*	279*

Figures in brackets include groups of less than 10 houses

* Primary cases were removed to hospital as soon as reported

We can use an arbitrary yard-stick to measure the infectivity, or infectiousness as it has been called by Dr Stocks and Miss Kain. Whichever factor is used for calculating the expected cases, the ratio of secondary cases to that factor will give an index of the infectivity. We have selected the ratio of the secondary cases to half the total population because the distribution according to the population appears to give the best fit among the untreated groups. Our index 'C' is the ratio $y / \frac{a+b}{2}$ multiplied by 1,000. Where a =the primary cases, b =the remaining persons in the same houses and y =the secondary cases.

Table XXXIII is a summary of Tables LIV to LVII (see Appendix). It gives the value of C for the various untreated and treated groups in the four epidemics. It is clear that in every instance there is a fall in the index of infectivity as between untreated and cases treated with bacteriophage.

Dr Persis Putnam has kindly pointed out that, though we have dealt with the extraction of cases dying on the first day of illness from the untreated group when considering mortality, we have not done so when considering infectivity. If cases dying on the first day are more dangerous, the analysis indicating a larger number of secondary cases in untreated households does not make a wholly justifiable comparison. If, however, the cases dying on the first day are abstracted from the untreated group (Sibsagar) the infectivity index is increased from 192 to 198. The group of 29 primary cases that died on the first day have an infectivity index of 141, which is below the 198 for the untreated cases which lived more than one day. The fulminating case, quickly bowled over, seems to have had less opportunity to spread infection. We need not suppose that the virus was less infective.

We have seen that the distributions by age, sex and caste were similar between treated and untreated groups in the same epidemics. These distributions, except in the small tea garden groups, showed no evidence of conscious or unconscious selection of cases. We know further that, in the village epidemics, no measures were possible for the isolation of cases of one group or another. As far as it is possible to judge, the susceptibility of the persons at risk and the conditions favouring transmission of the infection were alike in untreated and treated groups. Of the factors embraced by the term infectivity there remains, therefore, only the infective power of the primary case and, under these conditions, differences in the infectivity of the primary cases may be measured by C .

The earlier bacteriophage is given, if bacteriophage be a factor in reducing the infectivity, the more chance there is for a reduction in the value of C and we find in cases treated after 48 hours that C has a value intermediate between the high values in the untreated groups and the low value for the groups that had bacteriophage within 48 hours.

In the untreated groups in Sibsagar (Table LIV—see Appendix), where, as we have seen, the natural dissemination of bacteriophage seems to have had, week by week, a progressive effect on the mortality, we have likewise a progressive diminution in the index of infectivity. In the Darrang villages (Table LV—see Appendix) where the facilities for the dissemination of bacteriophage by a river were absent the reduction in the index of infectivity comes only late in the epidemic.

The groups where bacteriophage was not used are divided into village cases where there were no hospitals and no isolation and tea estate cases which were removed as soon as possible to an isolation hospital. The lowering of the index of infectivity in the tea estates has nothing to do with treatment, but is a measure of the reduction, by removal to a hospital, of the possibilities for infection in the house and of the protection by vaccination vigorously carried out in the tea gardens during the epidemic. In Darrang tea estate, group B, these measures seem less effective than in group A. We have shown in Part II that group A consisted of a main garden, its out-garden and a village lying between them, all under one superintendent. Group B consisted of five scattered tea estates, each of these was under a different management. The two groups are not comparable, for facilities for hospitalization were more likely to be quickly effective in group A than in group B.

The small vaccinated groups in the Sibsagar and Darrang village epidemics had an index of infectivity of 0 (Table XXXIII). When a case occurred in a vaccinated member of a family, it is reasonable to suppose that he was not the only vaccinated member of that family. As no roll had been kept, we cannot say how many of the vaccinated escaped infection. Further, these cases occurred near the end of the epidemic when natural distribution of bacteriophage was taking place. Both factors were in action. In the small tea-garden groups of 12 vaccinated cases the infectivity index is relatively high ($C=174$).

In the village groups which had been vaccinated and received bacteriophage within 48 hours (Table XXXIII), the values of C are 39 and 62. In the groups receiving only bacteriophage the corresponding figures are 56 and 66, and in the untreated groups 192 and 203. It may be that the lower values 39 and 62 measure the reductions of the index of infectivity brought about by the presence of vaccinated persons in the bacteriophage treated population.

We may express the results in a different way. If we take the value of C in untreated groups to be 193.9, the value calculated from the untreated in both Sibsagar and Darrang villages, then in 100 households of six persons, each having one primary case and five persons at risk, the number of secondary cases likely to arise if no treatment is given is — $\frac{300}{1,000} \times 193.9 = 58$

In a similar number of houses where bacteriophage has been given to the primary case within 48 hours and where the value of C is 58.5, we may expect 18 secondary cases, a reduction of 69 per cent in the secondary cases. It must be borne in mind that the primary bacteriophage cases treated within 48 hours had opportunities to infect before they received bacteriophage for, in these epidemics, the primary case only received bacteriophage when symptoms were well developed.

In Nowgong where bacteriophage is available in every village, and is being used by the villagers themselves for ordinary diarrhoeas and dysenteries whenever these occur, there is more chance of a patient seized with cholera getting bacteriophage earlier than there was in these epidemics. The value of C might be expected to be lower and the chances of secondary cases occurring much less. This, we believe, is the explanation of the remarkable abolition of cholera in Nowgong described in Part I.

That the Nowgong result was a chance occurrence is very unlikely from the consideration of the previous history of the district back as far as 1906. That it was not due to a general diminution of cholera in the Province has been shown by the comparison with the other districts in the same valley, and particularly with the neighbouring district of Darrang. As far as we can judge, it can be explained only by a lowering of infectivity by the early administration of bacteriophage.

As far as we have gone, d'Herelle's assertion that, under ordinary village conditions, cholera epidemics are often brought to an end by the development of and distribution of bacteriophage by natural agencies, has foundation, especially where conditions for the dissemination of bacteriophage are favourable.

These studies have convinced us that could we have bacteriophage at hand in every village in Assam, as it is now available in Nowgong and in Habiganj, the abolition of epidemic cholera in the Province is bound to follow.

SUMMARY

The work is a continuation of the researches already described (Morison, 1932).

PART I deals with the prevention of cholera by the administration of cholera-dysentery bacteriophage to all cases of diarrhoea, dysentery and suspected cholera in the villages by the villagers themselves.

The experimental area, Nowgong, a district of 3,898 square miles, with a population of 562,581 had a triennial death rate from cholera of 122.0 per 10,000 from 1906 to 1919. Between 1919 and 1929 the triennial death rate from cholera was 39.2 per 10,000 but epidemics still occurred with great regularity. Since 1929, epidemic cholera was altogether absent, the total deaths from cholera during the three years were 53, 47 and 27 respectively and the triennial death rate was 2.23 per 10,000. The first fall in the death rate in 1920 followed the great epidemic of 1919, compulsory vaccination of tea-garden coolies entering Assam and the use of vaccine in villages when cholera broke out. Its effect, seen in Nowgong, was more marked in the great tea-garden districts of Sibsagar and Lakhimpur. The absence of epidemic cholera after 1929 followed the distribution of bacteriophage to the villages along the Kalang river where cholera was prone to occur and the withdrawal of vaccination, at first partial and later complete. Such absence of cholera during the last three and a half years did not occur in the arbitrarily selected control area Habiganj, in the Surma Valley, nor in the more appropriate control districts adjoining Nowgong with which, previous to 1929, there was a high correlation in the deaths from cholera.

PART II—Outbreaks of cholera in four areas, each with its own peculiar conditions, are described.

PART III—Among 1,007 cases of cholera in two epidemics, 364 bacteriophage treated cases had a mortality of approximately 50 per cent of that of the untreated cases.

The comparisons are made in groups which, to tests of age, sex and caste, are homogeneous. The treatment was carried out under primitive village conditions and the majority of cases received only one dose of 2 c.c.

In one epidemic where conditions for the natural distribution of bacteriophage were present, the mortality of the untreated cases showed, week by week, a progressive fall. In another epidemic where facilities did not exist, the mortality remained high until the last week of the epidemic.

In these two epidemics, during successive weeks, the cases treated with bacteriophage showed a mortality which, in each case, approximated a constant level. The effect of bacteriophage was as great at the beginning of the epidemic as at the end.

The fallacies in estimating the value of any treatment of cholera have been discussed and allowed for.

Two epidemics where cases were taken to hospital and other forms of treatment were used are given for comparison.

PART IV --An outbreak in Sibsagar indicated that, when the first case in a house received bacteriophage early, fewer subsequent cases occurred in that house than when the first case received no bacteriophage. The data from 736 houses with a total population of 4,755 show that, in groups in all other respects alike, the number of subsequent cases in houses is significantly less when the first cases in these houses received bacteriophage. The reduction in the subsequent cases under these conditions for the whole data is about 69 per cent. Analysing the data in strictly comparable groups the divergence of the 'observed' from the 'expected' in bacteriophage treated groups in every instance is such that it is significant.

CONCLUSION

The results establish a sufficient probability in favour of a significant effect of the administration of bacteriophage to form a basis of practical policy in the treatment and prevention of cholera in villages.

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We have had the privilege of having the manuscript of this paper perused by Sir William Hamer, who, in his third Milroy lecture (1906) 'On Epidemic Disease in England—The Evidence of Variability and of Persistency of Type', put forth 'the conception of the bacillus as a kind of host with attached "enzymes"'—a prophetic dream of the bacteriophage, by Dr. Persis Putnam of the Rockefeller Foundation Statistical Department, by the Tropical Diseases Committee of the Royal Society, and, under their auspices, by five members of the Statistical Committee of the

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APPENDIX

ADDITIONAL TABLES

TABLES XXXIV to XLV—Monthly Death Rates for Districts

TABLES XLVI to LIII—Classified Mortality Tables of Cholera Outbreaks

TABLES LIV to LVII—Infectivity Tables

TABLE XXXIV.

GOALPARA DISTRICT

Monthly death rate per 10,000 of population

Year	January	February	March	April	May	June	July	August	September	October	November	December	Annual death rate per 10,000	Total annual deaths	Population
1906	14.96	5.61	3.74	8.42	42.26	3.16	6.69	2.81	0.54	0.18	3.92	4.96	107.25	2,982	278,038
1907	1.82	0.88	0.63	1.37	3.26	0.63	0.11	0.7	0.18	1.68	9.23	3.61	22.44	641	285,638
1908	1.26	0.58	0.89	3.68	1.57	1.91	1.43	0.68	0.55	0.03	0.07	0	12.64	371	293,569
1909	0.07	0.03	0.03	5.40	8.02	4.74	0.60	0.60	0	0.43	2.59	4.54	27.05	816	301,657
1910	2.58	0.45	1.19	3.68	10.26	9.10	3.65	0.94	0.61	3.42	19.81	21.62	77.30	2,396	309,967
1911	2.76	0.19	0.09	0	0.31	0.50	0.13	0	0.06	0.06	0	0.31	4.43	141	318,506
1912	0.55	0.37	0.64	0.15	3.77	8.73	4.23	0.34	1.13	0.34	6.68	10.11	37.04	1,209	326,382
1913	3.98	0.75	0.39	0.75	2.03	2.18	0.63	0	0.9	0.9	1.23	2.60	14.71	492	324,452
1914	0.67	0.41	0.29	0.67	0.35	0.20	0.06	0.03	0.03	0.18	1.84	4.76	9.48	325	342,722
1915	2.59	0.74	1.00	1.85	2.68	5.18	12.87	8.17	8.74	17.82	47.21	20.30	129.16	4,536	351,196
1916	6.39	0.36	0.39	0.92	10.75	8.25	4.36	1.06	1.47	1.80	7.70	8.86	52.32	1,883	359,880
1917	3.06	0.33	0.08	1.93	3.63	1.41	0.57	0.49	0.05	0.11	0.05	0.11	11.82	436	368,779
1918	0	0	0.03	0.29	0.85	0.53	0.19	0.05	2.17	5.35	11.41	17.91	38.77	1,465	377,898
1919	3.20	3.25	2.48	2.07	3.95	6.38	2.40	0.96	0.15	0.10	1.78	0.31	27.04	1,047	387,242
1920	0.58	0	0	0.23	0.15	0.18	0.03	0	0.08	0	0.05	0.05	1.34	53	396,817
1921	0	0.02	0	0.10	0.79	1.11	0.12	2.48	9.84	27.37	21.37	5.95	69.15	2,812	406,628
1922	1.62	0.24	0.02	1.09	0.68	1.11	0.17	0.39	0.12	0.07	0.05	0.05	4.90	202	412,584
1923	0	0.57	0.05	0.21	0.19	0.10	0.81	0.19	0.76	1.03	2.44	3.56	9.91	415	418,628
1924	0.54	0.05	0.05	0.26	1.39	7.82	9.58	2.21	3.13	2.17	11.82	10.29	49.30	2,094	424,760
1925	4.52	0.79	0	0.35	1.25	0.37	0.58	0	0.02	0.42	0.46	1.25	10.02	432	430,982
1926	0.21	0.43	0.94	5.85	16.95	10.15	5.05	0.75	3.13	0.07	0.02	0.02	43.59	1,906	437,295
1927	0.03	0.05	0.02	0.05	0.88	0.16	0	0	0.07	0.20	6.18	5.66	13.30	590	443,700
1928	1.91	0.73	0.38	1.62	2.13	2.75	2.13	0.71	0.78	4.33	11.2	10.33	39.00	1,756	450,199
1929	4.12	0.63	0.09	3.06	7.62	3.61	1	0.92	0.04	0.09	0.88	1.99	24.30	1,110	456,794
1930	0.73	0.06	0.24	0.39	0	0.04	0.25	0.11	0.35	2.80	10.92	9.10	24.94	1,156	463,485
1931	2.66	0.94	0.40	0.17	0	0.04	0.02	0.17	0.02	1.34	11.91	6.49	24.16	1,136	470,273
1932	1.59	0.38	0.38	0.10	0.19	0.04	0.54	0.04	0.08	0.02	0	0.08	3.46	165	477,162

TABLE XXXV
KANPUR DISTRICT

Monthly death rate per 10,000 of population

Year	January	February	March	April	May	June	July	August	September	October	November	December	Annual death rate per 10 000	Total annual deaths	Population
1906	11.45	7.13	15.09	38.19	04.08	34.55	6.23	1.70	4.43	5.99	7.05	2.56	198.46	12,465	628,089
1907	0.66	0.24	0.36	0.53	1.12	0.83	0.31	0.27	0.08	0.22	0.30	0.19	5.47	348	636,006
1908	0.73	0.92	1.68	8.01	9.84	8.68	5.54	1.29	0.50	0.39	0.16	0.03	37.76	2,432	844,014
1909	0.09	0.09	0.18	0.32	0.71	0.66	1.00	0.35	1.40	1.33	2.27	1.73	10.14	661	652,122
1910	1.82	0.36	0.39	1.20	1.15	7.51	4.06	3.56	6.15	26.41	14.78	6.21	76.6	5,058	660,332
1911	1.05	0	0.21	0.45	0.24	0.13	0.73	0.21	0.27	0.15	0.73	1.30	5.47	366	608,616
1912	0.15	0.10	0.72	0.93	1.36	1.98	1.48	1.92	4.46	6.02	10.74	12.40	42.25	2,863	677,582
1913	3.35	0.82	0.51	1.28	16.06	9.86	6.18	1.09	0.50	0.31	0.87	0.22	41.04	2,818	686,637
1914	0.27	2.46	1.19	0.49	0.65	0.59	2.79	1.37	0.46	0.14	0.39	0.33	11.12	774	695,812
1915	0.60	0.35	0.34	0.47	0.88	0.52	0.50	1.28	1.80	6.69	8.81	8.08	30.20	2,136	705,111
1916	0.98	0.88	0.50	0.73	9.12	3.09	1.97	0.78	0.41	0.31	0.87	2.41	21.52	1,538	714,534
1917	0.28	1.26	5.81	16.08	20.91	20.33	8.80	1.60	0.61	0.17	0.84	0.14	85.92	6,221	724,083
1918	0.45	0.42	0.26	1.27	2.22	0.26	0.44	0.19	0.38	0.20	1.31	1.95	8.80	652	733,759
1919	1.0	0.56	1.30	5.26	14.54	17.63	12.06	1.99	1.82	1.33	0.98	0.30	58.88	4,378	743,665
1920	0.05	0.05	0.40	0.44	0.32	0.11	0.12	0.07	0.09	0.12	0.19	0.08	2.03	153	753,501
1921	0.63	0.17	0.17	0.33	0.28	0.39	0.29	1.19	2.38	7.22	3.23	0.75	16.42	1,254	763,571
1922	0.17	0.32	2.08	17.86	30.62	13.90	9.60	3.55	1.29	0.23	0.36	0.12	80.4	6,292	782,009
1923	0.12	0.24	0.60	0.56	0.40	0.24	0.57	0.24	0.91	0.85	2.97	1.87	9.56	767	802,114
1924	0.16	0.12	1.19	9.05	12.19	22.53	13.16	2.68	2.59	0.83	0.90	0.38	65.77	5,407	822,109
1925	0.19	0.12	0.19	0.21	1.0	2.36	2.62	1.91	2.16	1.29	0.23	0.14	12.43	1,047	842,602
1926	0.14	0.10	0.42	7.16	22.80	18.54	5.19	2.17	1.05	0.27	0.87	0.08	58.78	5,076	863,606
1927	0.11	0.03	0.06	0.19	1.00	1.32	0.38	0.32	0.09	0.29	0.89	1.46	6.72	595	885,133
1928	0.3	0.04	0.23	0.24	0.39	0.24	0.08	0.09	0.04	0.13	1.08	0.41	3.27	297	907,200
1929	0.28	0.38	0.59	5.24	5.47	3.20	0.81	0.43	0.13	0.12	0.37	0.20	16.22	1,508	929,812
1930	0.07	0.08	0.18	0.99	1.0	0.40	0.98	0.63	1.28	1.25	1.0	1.79	9.65	920	952,990
1931	0.88	0.28	0.25	0.41	0.60	0.64	0.10	0.08	0.12	0.11	0.71	2.81	6.90	683	976,746
1932	0.98	0.56	1.20	6.01	3.33	3.03	3.48	2.34	1.45	0.16	0.06	0.01	21.00	2,162	1,001,090

TABLE XXXVI

DARRANG DISTRICT

Monthly death rate per 10,000 of population

Year	January	February	March	April	May	June	July	August	September	October	November	December	Annual death rate per 10,000	Total annual deaths	Population
1906	5.29	5.52	16.62	23.01	29.88	39.79	25.74	10.28	0.76	0.51	0.79	1.07	159.26	5,635	355,077
1907	0.25	1.17	1.14	1.11	1.00	0.42	1.50	1.06	0.22	0.31	0.72	1.36	10.13	361	359,352
1908	1.29	7.08	15.37	19.72	20.29	9.64	3.28	3.44	2.86	0.63	0.36	0.14	84.10	3,054	363,145
1909	0.08	0.22	0.68	0.87	2.67	3.78	2.91	1.17	0.44	0.27	0.68	2.10	15.87	583	367,247
1910	0.65	0.05	0.59	0.94	4.77	5.66	1.48	0.70	1.54	4.18	4.53	4.47	29.57	1,097	370,995
1911	0.8	0.48	0.13	0.35	1.76	0.91	0.53	0.13	0.24	0.27	0.16	0.08	5.83	219	375,591
1912	0.08	0.13	0.73	1.30	6.91	18.85	14.66	6.42	3.82	7.41	1.46	0.78	62.54	2,406	384,092
1913	0.23	0.08	0.58	0.89	3.20	4.70	1.60	0.63	0.71	0.35	0.08	0.10	13.15	518	384,013
1914	0.07	0.10	0.30	1.09	2.78	7.51	4.91	2.28	0.92	0.30	0.02	1.24	21.01	818	403,559
1915	0.17	0.22	0.39	1.69	2.47	0.99	0.75	0.82	2.10	1.28	2.18	3.51	16.57	685	413,338
1916	1.84	0.38	0.92	3.68	15.24	15.09	10.77	5.13	2.81	1.16	1.77	0.57	59.36	2,513	423,373
1917	0.14	0.14	0.51	1.57	14.78	9.78	2.88	0.44	0.37	0.16	1.25	3.97	35.98	1,350	433,611
1918	0.9	0.25	0.54	0.95	1.87	1.08	0.72	0.50	0.20	0.59	2.09	3.78	8.96	598	444,117
1919	4.18	2.81	9.59	17.57	20.27	12.44	7.96	3.12	0.90	0.86	0.26	0.59	80.55	3,664	451,877
1920	0.30	0.13	0.28	0.67	0.82	0.67	0.49	0.26	0.32	0.41	0.26	0.26	4.85	226	465,859
1921	0.23	0.13	0.19	0.80	1.17	1.30	0.69	0.73	0.34	0.23	0.15	0.57	6.52	311	477,188
1922	3.12	2.01	3.90	4.29	15.17	17.21	10.16	7.41	4.52	1.66	1.97	0.49	71.93	3,303	486,993
1923	0.12	0.28	0.14	0.44	0.50	0.42	0.32	0.44	0.26	0.36	0.28	0.08	3.66	182	496,959
1924	0.04	0.14	0.53	0.51	0.95	0.81	0.37	0.35	0.26	0.59	0.35	0.32	5.22	265	507,211
1925	0.08	0.14	0.01	0.70	7.03	6.43	1.76	0.54	0.23	0.25	0.23	0.14	17.56	909	517,623
1926	0.06	0.09	0.51	1.59	4.69	2.23	0.93	0.64	0.61	0.31	0.11	0.08	11.85	626	528,509
1927	0.09	0.07	0.19	0.30	0.80	2.37	1.39	1.52	2.97	4.88	2.93	1.34	18.85	1,016	539,123
1928	0.29	0.15	0.31	0.58	0.84	0.45	0.65	0.18	0.07	0.24	0.07	0.62	4.45	245	550,500
1929	0.25	0.02	0.78	0.16	1.98	1.00	0.93	0.07	0.07	0.11	0.16	0.11	5.63	316	561,505
1930	0.05	0.33	0.05	0.16	0.37	0.52	0.21	0.17	0.02	0.19	0.21	1.10	3.36	194	573,041
1931	0.42	0.12	0.24	0.17	0.67	0.10	0.29	0.03	0	0.07	0.17	1.64	3.93	230	584,917
1932	0.69	0.20	0.30	0.32	1.49	0.55	1.36	0.35	1.01	0.92	1.02	2.36	19.57	631	596,833

TABLE XXXVII

Nowgong District

Monthly death rate per 10,000 of population

Year	January	February	March	April	May	June	July	August	September	October	November	December	Annual death rate per 10,000	Total annual deaths	Population
1906	0	0	0.7	16.9	11.9	11.9	14.8	3.2	3.0	2.8	12.7	6.1	87.8	2,100	280,275
1907	0.3	0.1	0.14	0.2	0.25	0.04	0.04	0	0.07	0.18	0.18	0.25	1.8	50	281,127
1908	0.07	0.2	2.5	11.6	15.1	5.6	1.9	2.3	9.0	7.1	3.3	0.5	59.1	1,705	288,039
1909	0.11	0.03	0.5	0.4	0.14	0.8	0.2	0.07	0.1	0.11	0.1	0.11	3.0	89	292,914
1910	0.10	0.17	0.07	0.81	7.70	11.03	5.42	6.90	1.82	9.35	27.89	17.76	89.018	2,047	297,262
1911	1.79	0.23	0	0.06	0.09	0.27	0.27	0.13	0.03	0.2	0.3	0.11	3.51	106	301,655
1912	0	0	0.81	12.38	18.09	20.5	5.08	0.26	1.23	1.87	1.23	0	61.43	1,905	310,133
1913	0	0.03	1.91	0.78	4.42	0.85	3.23	0.56	6.40	5.05	5.05	0.19	28.63	913	318,860
1914	0	0	0.03	1.04	0.88	3.11	0.95	1.07	0.60	1.83	9.21	7.81	26.78	878	327,822
1915	5.61	6.97	17.39	22.31	10.18	0.56	0.53	2.91	1.78	7.09	11.39	1.09	96.82	3,293	337,025
1916	0.52	0.69	5.89	17.2	16.02	7.24	3.26	2.77	0.84	1.10	1.41	0.06	57.00	1,975	346,498
1917	0.06	0	2.13	0.39	0.25	0.59	0.11	0.17	0.33	0.16	1.09	0.11	5.17	181	350,237
1918	0.03	0	0.19	0.08	0.08	0.16	0.05	0.08	0.17	0.03	0.14	0.03	1.11	19	366,249
1919	0.24	0.32	1.04	1.65	10.57	14.05	3.93	3.45	3.21	6.06	3.08	0.10	47.99	1,907	376,547
1920	0.08	0.03	0.03	0.10	0.28	0.05	0.10	0.08	0.23	0.55	0	0.23	1.12	55	387,126
1921	0	0.03	0.03	0.15	0.25	0.06	0.10	0.38	0.11	0.21	5.70	8.17	15.45	615	398,007
1922	2.52	0.49	1.26	0.15	3.49	5.19	7.94	3.06	0.29	0.17	0.10	0	21.68	1,016	412,022
1923	0.02	0.05	0.54	0.12	0.14	0.12	0.02	0	0.14	0.23	0.16	0	1.62	69	426,530
1924	0.02	0.02	0.14	0.25	0.16	0.61	0.32	0.68	0.07	0.09	0.07	0.18	2.60	115	441,549
1925	0.74	0.24	0.20	0.98	3.74	7.07	3.06	1.60	0.18	0.98	7.02	5.47	31.28	1,130	457,007
1926	0.19	0.11	0.02	1.63	3.72	3.36	0.49	0.02	0.21	0.19	0.01	0.02	9.59	451	473,193
1927	0.02	0.08	0	0.27	2.72	0.78	0.49	0.37	4.70	6.29	5.23	10.96	31.89	1,562	499,855
1928	1.08	0.16	0.20	0.24	0.04	0.30	0.69	0.37	0.14	0.91	0.95	0.06	4.93	250	507,104
1929	0.08	0.36	1.18	2.93	1.30	1.03	0	0	0.21	0.04	0.01	0	7.16	376	521,960
1930	0.11	0.09	0.06	0.11	0.35	0	0.22	0.10	0	0.09	0.07	0	0.99	53	543,445
1931	0	0	0.12	0	0.07	0.02	0.02	0	0.04	0.21	0.14	0.16	0.78	44	562,581
1932	0	0.02	0	0.03	0.07	0.09	0.05	0.05	0	0.12	0.03	0	0.46	27	582,391

TABLE XLII

SOUTH SYLHET SUB-DIVISION

Monthly death rate per 10,000 of population

Year	January	February	March	April	May	June	July	August	September	October	November	December	Annual death rate per 10,000	Total annual deaths	Population
1906	6.10	2.11	2.16	0.86	1.53	0.99	0.76	0.13	0	0.20	0.71	4.12	19.68	774	393,315
1907	2.13	6.79	6.01	3.53	2.37	0.43	0.03	0	0	0.08	4.06	3.74	30.16	1,195	396,210
1908	2.33	1.68	3.38	13.20	11.75	4.43	0.63	0.23	0.30	0	0.05	0	37.98	1,516	399,126
1909	0	0.12	0.05	5.65	5.92	0.39	0.17	0	0.50	0	0.10	0.42	14.33	376	402,063
1910	0.44	0.10	0.02	0.17	0.35	0.05	0.02	0	0.72	7.46	4.37	2.91	16.62	673	405,021
1911	4.14	2.70	2.08	2.82	2.43	0.20	0	0	0.12	0	0.15	0.47	15.10	616	408,002
1912	1.64	0.27	0.88	1.56	1.30	0.54	0.19	0	0	0.05	0.02	0.12	6.55	268	408,993
1913	0.51	0.61	0.98	4.51	5.85	2.89	0.68	2.32	6.29	6.24	2.93	5.98	39.76	1,630	409,987
1914	2.97	1.07	0.46	0.36	0.49	0.10	0.02	0	0	0.02	0.32	0.71	6.52	268	410,982
1915	0.80	0.12	0.95	3.47	1.67	0.63	0.15	0.24	2.21	4.39	6.75	12.96	34.35	1,415	411,991
1916	6.20	1.38	1.07	1.69	1.14	0.29	0	0.10	0.05	0.05	0.02	0.05	12.03	497	412,982
1917	0	0.05	0.87	0.99	0.63	0.53	0.48	0.12	0.05	0	0	0.22	3.94	163	413,985
1918	0	0.02	0.24	1.71	3.88	0.63	0.58	0.07	0	2.02	9.98	12.84	31.98	1,327	414,990
1919	2.93	3.58	12.79	35.87	11.76	3.49	0.91	0.19	0	0	0.07	0.22	71.80	2,987	415,990
1920	0.67	0.05	0.09	0.24	0.09	0.02	0	0	0.02	0	0.02	0.09	1.32	55	417,008
1921	0	0.43	0.26	2.06	5.22	0.53	0.14	0	0	0.26	0.57	2.54	12.01	502	418,022
1922	1.47	0.71	0.90	6.12	17.40	6.29	1.00	0.14	0.09	0	0	0	34.14	1,438	421,242
1923	0.63	0.02	0.09	0	0.12	0.07	0.07	0.09	0.02	0.44	0.07	0.57	1.25	53	424,487
1924	3.87	1.76	1.51	0.77	2.62	1.17	0.14	0.02	0.09	0	5.61	5.61	17.23	737	427,757
1925	0.05	0	0.07	1.37	0.58	0.49	0.21	0.23	0.14	0	0	0	9.86	425	431,053
1926	1.05	1.14	1.05	0.55	0.30	0.46	0.05	0.30	0.16	0	0	0.51	2.35	102	434,373
1927	1.84	0.61	0.63	1.48	1.23	3.31	1.78	0.48	0.16	0.80	1.35	2.03	15.88	695	437,720
1928	1.48	0.83	0.61	1.25	1.20	0.77	0.41	0.20	0.18	0.29	1.18	2.04	10.61	468	441,091
1929	0.96	0.27	0.27	1.78	1.26	0.92	0.29	0.56	0.07	0.18	0.25	1.08	11.11	494	444,490
1930	1.02	0.33	0.73	2.02	3.59	0.20	0.11	0.04	0.02	0.22	0.54	1.59	5.22	524	447,913
1931	0.84	0.15	0.77	1.39	2.22	1.24	0.66	0.11	0.04	0.27	0.93	0.69	11.63	525	451,364
1932						0.68	0.21	0.02	0.02	0	0	0.04	6.35	289	454,841

TABLE XLIII
NORTH SYLHET SUB-DIVISION
Monthly death rate per 10,000 of population

Year	January	February	March	April	May	June	July	August	September	October	November	December	Annual death rate per 10,000	Total annual deaths	Population
1906	2.04	1.75	1.02	1.08	0.45	2.30	1.30	1.14	0.24	0.33	0.81	1.54	14.02	690	492,211
1907	0.92	1.99	2.33	2.51	0.78	0.22	0.10	0.69	0.04	0.06	1.04	2.35	12.80	628	498,309
1908	1.47	1.35	1.03	3.88	1.17	0.79	0.32	0.11	0	0	0.02	0.02	12.78	625	501,063
1909	0	0	0.29	7.44	6.75	1.35	0.66	0.27	0.25	0.14	0.33	0.78	20.57	1,051	511,005
1910	3.51	0.70	0.91	0.19	0.54	1.26	1.35	1.62	5.33	11.95	12.06	6.90	17.47	1,151	517,427
1911	3.78	1.70	0.88	1.11	0.38	0.31	0.65	0.76	0.14	0.17	1.36	8.09	19.35	1,014	521,931
1912	3.93	0.44	1.35	1.45	3.92	1.67	0.25	0.19	0	0	0.10	1.12	11.77	776	625,411
1913	1.20	0.51	1.18	0.61	0.80	1.20	1.75	4.25	14.22	12.96	1.46	1.63	14.45	2,312	526,895
1914	1.04	0.51	0.02	0.08	0.81	0.44	0.08	0.06	0.08	0.42	1.04	0.72	5.28	2,279	528,383
1915	0.77	0.77	0.49	0.32	0.45	0.28	0.43	1.87	7.80	13.27	10.91	11.04	51.50	2,898	529,877
1916	2.41	1.06	0.81	0.23	0.08	0.30	0	0.04	0.17	0	0.02	0.04	5.74	305	531,374
1917	0	0.06	2.51	5.48	4.84	1.29	0.32	0	0	0.02	0	0.21	11.73	785	532,875
1918	0.13	0.09	0.37	0.17	0.90	0.30	0.21	0.52	0.80	3.78	12.50	9.54	29.29	1,505	534,380
1919	2.51	2.22	4.03	12.33	10.56	3.27	1.21	0.67	0.56	0.45	2.89	2.99	13.72	2,343	535,890
1920	1.49	0.37	0.30	0.07	0.07	0.22	0	0.02	0	0	0.19	0.04	2.87	154	537,404
1921	0.10	1.13	1.0	1.43	8.46	3.77	1.58	1.11	0.24	0.96	4.66	4.66	31.19	1,681	539,922
1922	1.46	0.39	0.26	0.20	2.45	1.97	0.55	0.07	0.37	0.06	0	0.02	7.79	423	542,948
1923	0.08	0.22	0.42	0.17	0.34	0.24	0.44	0.24	0.04	0	0.05	0.05	2.76	159	547,004
1924	1.71	1.29	1.78	4.92	5.59	3.18	1.09	0.53	0.11	2.78	4.48	3.07	30.54	1,083	551,090
1925	1.15	0.18	0.54	0.14	0.05	0.11	0.05	0	0	0	0.02	0	2.25	125	555,206
1926	0.04	0.21	0.27	1.00	5.33	4.76	0.82	0.45	0.18	0	0.02	0	13.34	746	559,354
1927	1.07	1.06	0.67	0.80	0.64	0.39	0.80	1.90	1.05	2.20	5.39	6.89	23.16	1,322	563,552
1928	2.20	0.67	0.33	0.80	1.14	0.97	0.21	0.05	0.09	1.80	0.07	0.02	6.36	361	567,742
1929	1.08	1.52	0.51	0.42	0.51	0.37	0.38	0.65	0.68	1.80	2.92	2.81	12.75	720	571,983
1930	2.38	0.61	0.52	0.05	0.09	0.42	0.36	0.21	0.10	0.16	4.91	3.07	12.93	745	576,265
1931	0.84	0.26	0.34	0.45	0.96	2.38	0.95	0.17	0.40	0.24	1.64	1.84	10.17	608	580,590
1932	0.77	0.72	0.21	0.24	0.19	0.15	0.12	0.02	0.02	0	0.02	0	2.44	113	584,897

TABLE XLIV

KARIMGANJ SUB-DIVISION

Monthly death rate per 10,000 of population

	January	February	March	April	May	June	July	August	September	October	November	December	Annual death rate per 10,000	Total annual deaths	Population
1906	1.01	0.18	0.23	1.95	1.99	1.24	0.92	0.11	0.09	0	0	0.16	7.79	340	436,484
1907	0.27	1.02	0.20	0.11	0.48	0.18	0.14	0	0	1.40	5.77	5.88	15.46	683	441,884
1908	1.00	2.03	4.83	8.14	8.81	2.53	0.22	0.04	0	0	0	0	30.60	1,369	447,351
1909	0	0	0.79	10.58	5.21	0.44	0.22	0.09	0.33	0.20	0.86	0.44	19.17	968	452,885
1910	0.09	0.04	0.15	0	0	0.07	0.09	0.39	1.61	4.38	2.12	0.52	9.88	453	458,487
1911	0.69	0.17	0.09	1.08	0.71	1.25	0.47	0.93	1.40	1.29	1.64	3.19	12.91	599	464,139
1912	1.74	0.43	0.21	2.81	4.64	2.66	0.34	0.04	0.02	0.06	0.79	1.61	15.57	725	465,745
1913	2.12	0.92	0.26	0.21	0.47	0	1.03	6.23	7.30	7.55	4.99	1.33	32.40	1,514	467,336
1914	1.07	0.13	0.11	0.13	0.15	0.19	0	0	0	0	0.09	0	1.86	87	468,933
1915	0	0	0.02	0	0.28	0.30	0.21	1.79	4.27	12.69	15.47	7.94	42.76	2,012	470,536
1916	1.33	0.30	0.30	0.51	0.72	0.19	0	0.06	0	0.06	0	0.02	3.49	165	472,142
1917	0	0.02	0.15	0.34	0.51	0.72	0.44	0.02	0.02	0.02	0	0.02	2.26	107	473,757
1918	0.02	0.02	0.08	0.08	0.55	1.47	0.95	0.78	1.72	7.40	8.34	8.29	29.91	1,422	475,375
1919	6.06	3.27	2.96	10.94	8.18	5.24	1.76	0.94	0.02	0.48	0.04	0.31	40.21	1,918	477,000
1920	0.21	0	0.02	0	0.06	0.10	0.13	0.10	0.02	0.04	0.06	0	0.75	36	478,630
1921	0	0.01	2.74	8.09	10.95	2.26	0.65	0.08	1.23	2.84	2.63	3.31	34.81	1,666	480,265
1922	1.39	0.38	0.52	0.95	0.79	0.52	0.25	0.70	0.14	0.08	0.19	0.02	6.33	306	483,122
1923	0	0	0.08	0.43	0.27	0.14	0.12	0.04	0.06	0	0.02	0.27	1.44	70	486,000
1924	0.35	2.60	5.15	9.18	10.10	4.68	1.02	0.18	0.14	0.25	0.31	0.29	34.26	1,675	488,893
1925	0.73	0.63	1.06	0.22	0.20	0.20	0	0	0	0.04	0	0	3.09	152	491,804
1926	0	0.18	0.06	0.26	0.67	1.17	0.75	0.20	0.02	0	0	0.04	3.36	166	494,732
1927	0.38	1.87	2.59	2.45	2.23	2.13	1.75	1.35	0.76	1.09	1.67	4.84	23.11	1,150	497,677
1928	2.52	0.78	0.20	0.82	2.34	0.98	0.26	0.10	0.10	0.14	0.14	0.48	8.87	144	500,640
1929	0.16	0.32	0.30	0	0.16	0.18	0.46	1.03	0.81	1.29	0.87	1.96	7.51	378	503,610
1930	0.30	0.04	0.10	0.06	0.12	0.28	0.06	0	0	0.02	0.04	0.26	1.26	64	506,618
1931	0.01	0.08	0.06	0.14	0.20	0.88	1.02	2.12	2.85	2.24	1.23	1.24	12.13	618	509,634
1932	0.90	0.60	0.04	0.06	0.18	0.10	0	0	0.02	0	0.02	0.04	1.95	100	512,668

TABLE XLV

CACHAR DISTRICT

Monthly death rate per 10,000 of population

Year	January	February	March	April	May	June	July	August	September	October	November	December	Annual death rate per 10,000	Total annual deaths	Population
1906	0.51	2.57	2.78	3.87	5.21	9.01	1.87	1.71	0.31	0.31	0.19	0.1	37.85	1,809	177,986
1907	0.79	0.35	0.54	0.37	0.27	0.27	0.19	0.23	0.04	0.10	0.17	0.33	12.80	617	182,209
1908	0.74	0.60	0.17	16.03	15.81	6.6	1.05	0.19	0.11	0.08	0.08	0.08	11.67	2,173	186,169
1909	0.12	0.02	0.24	1.57	1.25	3.93	0.75	0.18	0.35	0.18	0.55	3.08	15.22	717	190,766
1910	0.71	0.20	0.20	0.91	0.79	2.18	1.81	0.97	0.55	0.14	0.57	1.66	11.31	560	195,101
1911	2.66	2.62	4.58	3.18	5.74	4.18	0.94	0.60	0.20	0.10	0.16	0.78	26.04	1,302	199,175
1912	1.27	1.00	2.59	5.75	6.73	3.16	0.52	0.94	0.16	0.2	0.14	0.28	23.03	1,157	202,380
1913	0.12	0	0.26	0.12	0.34	0.07	0.97	3.27	7.18	10.83	5.64	3.16	32.65	1,650	205,392
1914	1.00	0.08	0.06	0.16	0.47	0.43	0.02	0.10	0.10	0.10	0.04	0	2.56	130	208,241
1915	0.02	0.12	0.12	0.22	1.76	0.76	0.20	0.10	0.20	0.88	13.87	11.87	30.11	1,539	211,197
1916	3.15	0.99	1.03	1.30	2.14	0.78	0.21	0.06	0.02	0.06	0.12	0.10	9.96	512	514,170
1917	0.04	0.02	0.23	0.18	0.93	0.52	0.27	0.15	0.15	0.15	0.17	0.06	3.19	165	517,161
1918	0.04	0.02	0.23	0.42	0.38	0.94	0.56	0.69	1.06	2.31	11.02	13.50	31.16	1,621	520,169
1919	3.71	5.56	14.09	22.38	8.20	4.28	1.82	0.34	0.11	0.25	0.04	0.04	60.82	3,182	523,195
1920	0.04	0.04	0.02	0.10	0.38	0.29	0.23	0.15	0.10	0.17	0.04	0.04	1.58	83	526,238
1921	0	0.11	0.43	3.89	14.38	5.21	0.21	0.76	0.17	0.11	0.47	1.91	27.06	1,164	529,301
1922	2.1	1.09	1.35	1.5	3.84	2.1	1.76	0.04	0.06	0.11	0.02	0.08	14.35	705	533,286
1923	0.06	0.22	0.37	1.02	0.82	0.6	0.15	0.07	0.09	0.04	0	0.06	3.55	191	537,301
1924	0.61	1.55	5.84	16.2	17.47	8.11	2.12	0.18	0.13	0.02	0.37	0.20	24.77	1,341	541,346
1925	0.24	0.40	0.70	1.87	0.37	0.35	0.06	0.09	0.06	0.06	0.04	0.04	1.22	230	545,422
1926	0.04	0.04	0.09	0.53	1.27	0.38	0.04	0.05	0.09	0	0	0	2.53	139	549,528
1927	0.23	0.89	3.00	2.94	1.86	3.45	2.28	0.90	0.27	0.27	0.61	2.96	19.67	1,089	553,665
1928	2.20	0.11	0.20	1.00	1.34	0.56	0.07	0.05	0.02	0.11	0	0.04	6.53	164	557,834
1929	0	0.11	0.09	0.07	0.27	0.27	1.05	0.44	0.09	0.09	0.18	0.41	3.06	172	562,034
1930	0.19	0.04	0.12	0.46	0.34	0.19	0.02	0.04	0.02	0.02	0	0.02	1.45	92	566,265
1931	0	0	0.07	0.11	0.26	0.05	0.80	1.02	0.31	0.35	0.32	0.75	1.15	237	570,531
1932	0.40	0.33	0.14	0.05	0.07	0.03	0	0.03	0	0.05	0	0	1.11	64	574,827

TABLE XLVI

Mortality of cases according to age and period

Sibsagar

Groups according to treatment of cases	Age groups	July 19 to 25		July 26 to Aug 1		Aug 2 to 8		Aug 9 to 15		Aug 16 to 22		Aug 23 on		TOTALS		
		R	D	R	D	R	D	R	D	R	D	R	D	R	D	M
No TREATMENT	0-5	1	4	5	7	10	17	16	13	4	4			36	45	55.6
	6-10	6	6	9	13	12	18	12	9	5	2	2	1	46	40	51.6
	11-20	1	2	10	11	29	17	17	2	8	0			65	32	33.0
	21-30	1	5	4	7	18	12	11	13	3	2	3	3	40	42	51.2
	31-40	0	1	1	2	7	5	2	3	2	0	0	2	12	13	52.0
	41-50			0	2	2	2	3	2			1	1	6	7	53.8
	51-60	0	1	0	0	1	1	1	0					2	2	50.0
	61-70			0	1			0		1				0	2	100.0
	TOTALS	9	19	29	43	79	72	62	43	22	8	6	7	207	192	48.12
	Mortality	67.85		59.72		47.68		40.95		26.67		53.85		48.12		
BACTERIOPHAGE WITHIN 48 HOURS	0-5			2	0	7	4	6	3	2	1			17	8	32.0
	6-10			1	2	13	2	4	1	2	0	2	0	22	5	18.5
	11-20			3	2	11	4	6	1	5	1	2	1	27	9	25.0
	21-30			0	1	6	1	4	2	3	0	1	1	14	5	26.3
	31-40			1	0	4	0	3	0	1	0	1	1	10	1	9.1
	41-50					1	0	2	0					3	0	0
	51-60							0	0					0	0	0
	61-70					0	1	1	0			1	0	2	1	33.3
	TOTALS			7	5	42	12	26	7	13	2	7	3	95	29	23.39
	Mortality			41.6		22.2		21.2		13.3		30.0		23.39		

R —Recovered D —Died M —Mortality percentage

TABLE XLVI—*contd*

Groups according to treatment of cases	Age groups	July 19 to 25		July 26 to Aug 1		Aug 2 to 8		Aug 9 to 15		Aug 16 to 22		Aug 23 on		TOTALS		
		R	D	R	D	R	D	R	D	R	D	R	D	R	D	M
BACTERIOPHAGE WITHIN 18 HOURS, VACCINATED PREVIOUSLY	0-5					2	0	3	0					5	0	0
	6-10					5	1	2	1	3	0	1	0	11	2	15.4
	11-20					5	2	3	0	2	2	1	0	11	4	26.6
	21-30					1	0					1	0	2	0	0
	31-40							1	3					1	3	75.0
	41-50					1	0							1	0	0
	51-60							1	0					1	0	0
	61-70															0
	TOTALS					14	3	10	4	5	2	3	0	32	9	22.0
	Mortality							17.6	28.6	28.6		0		22.0		
BACTERIOPHAGE AFTER 18 HOURS	0-5	1	1	1	0	1	0	3	0	2	0			8	1	12.5
	6-10			6	1	8	4	2	1	1	0	1	0	18	6	25.0
	11-20	1	0	1	1	8	2	3	2	0	1			13	6	31.5
	21-30	1	0	3	1	5	2	1	2	1	0			11	5	31.25
	31-40					1	0	2	0			2	0	5	0	0
	41-50			1	0									1	0	0
	51-60															0
	TOTALS	3	1	12	3	23	8	11	5	4	1	3	0	56	18	24.3
	Mortality		25		20		25.8		31.25		20		0		24.3	

R—Recovered D—Died M—Mortality percentage

TABLE XLVI—*concl'd*

Groups according to treatment of cases	Age groups	July 19 to 25		July 26 to Aug 1		Aug 2 to 8		Aug 9 to 15		Aug 16 to 22		Aug 23 on		TOTALS		
		R	D	R	D	R	D	R	D	R	D	R	D	R	D	M
BACTERIOPHAGE AFTER 48 HOURS, VACCINATED PREVIOUSLY	0-5															0
	6-10					2	0	2	0					4	0	0
	11-20							2	0			1	0	3	0	0
	21-30															0
	31-40							1	0					1	0	0
	41-50															0
	51-60															0
	61-70															0
	TOTALS					2	0	5	0			1	0	8	0	0
VACCINATED PREVIOUSLY	0-5					1	1	3	2	2	1			6	4	40 0
	6-10							2	1	0	1	4	0	6	2	25 0
	11-20					1	0			1	0			2	0	0
	21-30							4	1	2	0			6	1	14 0
	31-40									1	0			1	0	0
	41-50					0	1					1	0	1	1	50 0
	51-60															0
	61-70															0
	TOTALS					2	2	9	4	6	2	5	0	22	8	26 7
	Mortality						50 0		30 8		25		0		26 7	.

R —Recovered

D —Died

M —Mortality percentage

TABLE XLVII

Mortality of cases according to age and sex.

Sibsagai

Groups according to treatment of cases	Age groups	MALE			FEMALE			TOTALS		
		Recovered	Died	Mortality	Recovered	Died	Mortality	Recovered	Died	Mortality
NO TREATMENT	0-5	22	21	45.8	14	24	63.2	36	45	55.6
	6-10	20	27	57.4	26	22	45.8	46	49	51.6
	11-20	29	16	35.6	36	16	30.8	65	32	33
	21-30	15	11	73.3	25	31	55.4	40	40	51.2
	31-40	5	11	68.7	7	2	28.6	12	13	52.0
	41-50	6	6	50.0		1	100.0	6	7	53.8
	51-60	2	1	33.3		1	100.0	2	2	50.0
	61-70		2	100.0			0		2	100.0
	TOTALS	99	95	48.97	108	97	47.32	207	192	48.12
BACTERIOLOGIC WITHIN 48 HOURS	0-5	9	2	18.2	8	6	42.8	17	8	32.0
	6-10	12	1	7.7	10	4	28.6	22	5	18.5
	11-20	11	2	15.3	16	7	30.4	27	9	25.0
	21-30	5	2	28.5	9	3	25.0	14	5	26.3
	31-40	7	1	12.5	3		0	10	1	9.1
	41-50	3	0	0			0	3		0
	51-60		1	100.0					1	100.0
	61-70	1		0	1	0	0	2	0	0
	TOTALS	48	9	15.8	47	20	29.8	95	29	21.37

TABLE XLVII—*contd*

Groups according to treatment of cases	Age groups	MALE			FEMALE			TOTALS		
		Recovered	Died	Mortality	Recovered	Died	Mortality	Recovered	Died	Mortality
BACTERIOPHAGE WITHIN 48 HOURS, VACCINATED PREVIOUSLY	0-5	3			2			5		0
	6-10	5	1		6	1		11	2	15.4
	11-20	4	4		7			11	4	26.6
	21-30	1			1			2		0
	31-40	1	3					1	3	7.5
	41-50				1			1		0
	51-60	1						1		0
	61-70									0
	TOTALS	15	8	34.8	17	1	5.5	32	9	15.8
BACTERIOPHAGE AFTER 48 HOURS.	0-5	4	1		4			8	1	12.5
	6-10	14	2		4	4		18	6	25.0
	11-20	5	3		8	3		13	6	31.5
	21-30	3	4		8	1		11	5	31.25
	31-40	2			3			5		0
	41-50	1						1		0
	51-60									0
	61-70									0
	TOTALS	29	10	25.6	27	8	22.9	56	18	24.3

TABLE XLVII—concl'd

Groups according to treatment of cases	Age groups	MALE			FEMALE			TOTALS		
		Recovered	Died	Mortality	Recovered	Died	Mortality	Recovered	Died	Mortality
BACTERIOLOGICAL AFTER 19 HOURS, VACCINATED PREVIOUSLY	0-5									0
	6-10	3			1			4		0
	11-20				3			3		0
	21-30									0
	31-40	1						1		0
	41-50									0
	51-60									0
	61-70									0
	TOTALS	4		0	4		0	8		0
VACCINATED PREVIOUSLY.	0-5	4	1		2	3		6	4	40
	6-10	5			1	2		6	2	25
	11-20	1			1			2		0
	21-30	3			3	1		6	1	14
	31-40				1			1		0
	41-50	1	1					1	1	50
	51-60									0
	61-70									0
	TOTALS	14	2	12.5	8	6	42.9	22	8	26.7

TABLE XLVIII.

*Mortality of cases according to age and period**Dairang villages*

Groups according to treatment of cases	Age groups	Oct 16 to Nov 11		Nov 12 to 18		Nov 19 to 25		Nov 26 to Dec 2		Dec 3 to 9		Dec 10 to 16		Dec 17 to Jan 6		TOTALS		
		R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	M
NO TREATMENT	0-5			0	1	2	5			1	2	0	1	0	1	3	10	76.9
	6-10			0	3	3	0	1	3	1	2	1	2		.	6	10	62.5
	11-20			0	2	1	6	1	4	2	2	0	7			4	21	84.0
	21-30	0	1	1	4	1	6	2	5	0	2	0	3	1	1	5	22	81.5
	31-40	0	1	0	2	1	5	0	8	0	7	0	2	0	2	1	27	96.4
	41-50	0	2	0	1	1	3	0	2	1	2	0	1	1	1	3	12	80.0
	51-60	0	1	1	0	0	3	0	2	0	1	0	2	0	1	1	10	90.9
	61-70			0	1			0	1	0	1					0	3	100.0
	TOTALS	0	5	2	14	9	28	4	25	5	19	1	18	2	6	23	115	83.3
	Mortality	109		87.5		75.7		86.2		79.2		94.7		75.0		83.3		
BACTERIOPHAGE WITHIN 48 HOURS	0-5							3	0	1	1					4	1	20
	6-10									1	1	0	1			1	2	66
	11-20							1	3	1	1	3	1	1	0	6	5	45
	21-30					1	0	4	2	7	3	1	3	1	2	14	10	42
	31-40							2	1	3	4	1	0	1	1	7	6	46
	41-50							0	1	3	1	0	2	0	1	3	5	62
	51-60							0	1	1	0	1	0			2	1	33
	61-70																	0
	TOTALS					1	0	10	8	17	11	6	7	3	4	37	30	44.5
	Mortality	.				0		44		39		54		57		44.5		

R —Recovered D —Died. M —Mortality percentage

TABLE XLVIII—*contd*

Groups according to treatment of cases	Age groups	Oct 16 to Nov 11		Nov 12 to 18		Nov 19 to 25		Nov 26 to Dec 2		Dec 3 to 9		Dec 10 to 16		Dec 17 to Jan 6		TOTALS		
		R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	M
BACTERIOPHAGE WITHIN 18 HOURS, VACCINATED PREVIOUSLY	0-5											1	0	1	0	2	0	0
	6-10								0	1	1	0	1	0	2	1	33	
	11-20							0	1	0	1	1	1		1	3	43	
	21-30																0	
	31-40					1	0	1	0	1	0	0	2	1	0	6	2	25
	41-50							1	1			2	0			3	1	25
	51-60																	0
	61-70																	0
	TOTALS					1	0	2	2	3	2	8	3	3	0	17	7	29.2
	Mortality						0		50		40		27		0		29.2	
BACTERIOPHAGE AFTER 18 HOURS	0-5					1	0									1	0	0
	6-10					0	1			1	0	0	1			1	2	66
	11-20			1	1	1	0			0	1	0	1			2	3	60
	21-30					2	3	1	1	0	1	1	0			4	5	55.5
	31-40					2	0			2	0			0	1	4	1	20
	41-50					0	1	1	0			1	0			2	1	33
	51-60																	0
	61-70																	0
	TOTALS			1	1	6	5	2	1	3	2	2	2	0	1	14	12	46.1
	Mortality				50		45		33		40		50		100		46.1	

R—Recovered D—Died M—Mortality percentage

TABLE XLIX—*contd*

Groups according to treatment of cases	Age groups	MALE			FEMALE			TOTALS		
		Recovered	Died	Mortality	Recovered	Died	Mortality	Recovered	Died	Mortality
BACTERIOPHAGE WITHIN 48 HOURS, VACCINATED PREVIOUSLY	0-5	1			1			2		0
	6-10	1	1		1			2	1	33
	11-20		2		4	1		4	3	43
	21-30									0
	31-40	2	1		4	1		6	2	25
	41-50	3				1		3	1	25
	51-60									0
	61-70									0
	TOTALS	7	4	36	10	3	23	17	7	29 2
BACTERIOPHAGE AFTER 48 HOURS	0-5	1						1		0
	6-10	1	2					1	2	66
	11-20	2	2			1		2	3	60
	21-30	2			2	5		4	5	55
	31-40	4	1					4	1	20
	41-50	1	1		1			2	1	33
	51-60									0
	TOTALS	11	6	35	3	6	67	14	12	46 1
ESSENTIAL OIL TREATMENT	0-5				1			1		0
	6-10	1						1		0
	11-20	3			.	1		3	1	25
	21-30	4	1		2	3		6	4	40
	31-40	3	2		1			4	2	33
	41-50	1	1		1	1		2	2	50
	51-60	1	.			1		1	1	50
	61-70					.			.	0
	TOTALS	13	4	23 5	5	6	54 5	18	10	35 7

TABLE XLIX—*contd*

Groups according to treatment of cases	Age groups	Males			Females			Totals		
		Recovered	Died	Mortality	Recovered	Died	Mortality	Recovered	Died	Mortality
ESSENTIAL OIL TREATMENT, VACCINATED PREVIOUSLY	0-5		1						1	100.0
	6-10				2			2		0
	11-20	1			1			2		0
	21-30	1			2			3		0
	31-40				1			1		0
	41-50					1			1	100.0
	51-60									0
	61-70									0
	TOTALS	2	1	33	6	1	14	8	2	20.0
VACCINATED PREVIOUSLY	0-5				1	2		1	2	0
	6-10	1	1			1		1	2	0
	11-20					1			1	0
	21-30	1			3			4		0
	31-40	1	1					1	1	0
	41-50		1						1	0
	51-60					1			1	0
	61-70									0
	TOTALS	3	3	50.0	4	5	55.0	7	8	53.3

TABLE L

*Mortality of cases according to age and period**Darrang tea estates, 'A' group*

Groups according to treatment of cases	Age groups	Sept 13 to 29			Sept 30 to Oct 6			Oct 7 to 13			Oct 14 to 26			TOTALS		
		R	D	M	R	D	M	R	D	M	R	D	M	R	D	M
No TREATMENT	0-5							0	4					0	4	
	6-10				0	1								0	1	
	11-20				0	1		0	1					0	2	
	21-30				0	1								0	1	
	31-40															
	41-50				0	2								0	2	
	51-60															
	61-70															
	TOTALS				0	5	100	0	5	100				0	10	100
HYPERTONIC SALINE AND PERMANGANATE TREATMENT	0-5			8				0	1					0	1	
	6-10															
	11-20				2	2		3	1					5	3	
	21-30	1	0					5	1		1	0		7	1	
	31-40	1	0		4	3					0	1		5	4	
	41-50				1	1		0	1					1	2	
	51-60							1	0					1	0	
	61-70							1	1					1	1	
	TOTALS	2	0	0	7	6	46 1	10	5	33 3	1	1	0	20	12	37 5

R—Recovered D—Died M—Mortality percentage

TABLE L—concl'd

Groups according to treatment of cases	Age groups	Sept 13 to 29			Sept 30 to Oct 6			Oct 7 to 13			Oct 14 to 26			TOTALS		
		R	D	M	R	D	M	R	D	M	R	D	M	R	D	M
HYPERTONIC SALINE AND PERMANGANATE TRIATMENT, VACCINATED PREVIOUSLY	0-5										1	0		1	0	
	6-10															
	11-20							0	1		3	1		3	2	
	21-30							1	0		1	1		2	1	
	31-40										4	1		4	1	
	41-50							2	1		3	1		5	2	
	51-60															
	61-70															
	TOTALS							3	2	40	12	4	25	15	6	28 6
PERMANGANATE TRIATMENT	0-5				4	1		2	1					6	2	
	6-10							1	0					1	0	
	11-20															
	21-30				1	0		1	0					2	0	
	31-40							2	0					2	0	
	41-50															
	51-60															
	61-70							0	1					0	1	
	TOTALS				5	1	20	6	2	25				11	3	21 4
VACCINATED PREVIOUSLY	0-5															
	6-10															
	11-20							0	1					0	1	
	21-30										0	2		0	2	
	31-40							0	1		0	1		0	2	
	41-50															
	51-60							0	1					0	1	
	61-70															
	TOTALS							0	3	100	0	3	100	0	6	100

R—Recovered D—Died M—Mortality percentage

TABLE LI

Mortality of cases according to age and sex

Darrang tea estates, 'A' group

Groups according to treatment of cases	Age groups	MALE			FEMALE			TOTALS		
		R	D	M	R	D	M	R	D	M
No TREATMENT.	0-5	0	2		0	2		0	4	
	6-10	0	1					0	1	
	11-20	0	1		0	1		0	2	
	21-30	0	1					0	1	
	31-40									
	41-50	0	1		0	1		0	2	
	51-60									
	61-70									
	TOTALS	0	6	100	0	4	100	0	10	100
HYPERTONIC SALINE AND PERMANGANATE TREATMENT	0-5	0	1					0	1	
	6-10									
	11-20	4	2		1	1		5	3	
	21-30	6	1		1	0		7	1	
	31-40	4	0		1	4		5	4	
	41-50	1	1		0	1		1	2	
	51-60				1	0		1	0	
	61-70	1	1					1	1	
	TOTALS	16	6	27.3	4	6	60	20	12	37.5

R—Recovered D—Died M.—Mortality percentage

TABLE LI—*concl'd*

Groups according to treatment of cases	Age groups	MALF			1 FMALF			TOTALS		
		R	D	M	R	D	M	R	D	M
HYPERTONIC SALINE AND 11 PERMANGANATE TREATMENT, VACCINATED PREVIOUSLY	0-5	1	0					1	0	
	6-10									
	11-20				3	2		3	2	
	21-30	2	1					2	1	
	31-40	1	0		3	1		4	1	
	41-50	4	1		1	1		5	2	
	51-60									
	61-70									
	TOTALS	8	2	20	7	4	36.4	15	6	28.6
PERMANGANATE TREATMENT	0-5	6	1		0	1		6	2	
	6-10	1	0					1	0	
	11-20									
	21-30	2	0					2	0	
	31-40	2	0					2	0	
	41-50									
	51-60									
	61-70	0	1					0	1	
	TOTALS	11	2	15.4	0	1	100	11	3	21.4
VACCINATED PREVIOUSLY	0-5									
	6-10									
	11-20	0	1					0	1	
	21-30	0	1		0	1		0	2	
	31-40									
	41-50				0	2		0	2	
	51-60				0	1		0	1	
	61-70									
	TOTALS	0	2	100	0	4	100	0	6	100

R —Recovered D —Died M —Mortality percentage

TABLE LII.

*Mortality of cases according to age and period.**Darrang tea estates, 'B' group*

Groups according to treatment of cases	Age groups	Oct 16 to Nov 11		Nov 12 to 18		Nov 19 to 25		Nov 26 to Dec 2		Dec 3 to 9		Dec 10 to 16		Dec 17 to Jan 6		TOTALS		
		R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	M
No TREATMENT	0-5									0	1	0	2			0	3	
	6-10																	
	11-20													0	2	0	2	
	21-30									0	1	0	2	0	1	0	4	
	31-40									0	1			0	1	0	2	
	41-50																	
	51-60																	
	61-70																	
	TOTALS									0	3	0	4	0	4	0	11	100
HYPERTONIC SALINE AND PERMANGANATE TREATMENT	0-5									1	0	1	1			2	1	
	6-10									0	2	0	2			0	4	
	11-20									2	0	2	0	0	1	4	1	
	21-30									1	1	0	3	1	1	2	5	
	31-40									0	1	3	0	0	1	3	2	
	41-50											2	3	0	1	5	4	
	51-60																	
	61-70																	
	TOTALS									4	4	8	9	1	4	13	17	56.6

R.—Recovered D—Died M—Mortality percentage

TABLE LII—*concl'd*

Groups according to treatment of cases	Age groups	Oct 16 to Nov 11		Nov 12 to 18		Nov 19 to 25		Nov 26 to Dec 2		Dec 3 to 9		Dec 10 to 16		Dec 17 to Jan 6		TOTALS		
		R	D	R	D	R	D	R	D	R	D	R	D	R	D	R	D	M
HYPERTONIC SALINE AND PERMANENT TRIATMIN, VACCINATED PREVIOUSLY	0-5							0	1			2	1	1	0	3	2	
	6-10													1	0	1	0	
	11-20											1	0			1	0	
	21-30							0	1					1	0	1	1	
	31-40							0	1	0	2	1	1			1	4	
	41-50											0	1	2	0	2	1	
	51-60																	
	61-70																	
TOTALS								0	3	0	2	4	3	5	0	9	8	47.05
VACCINATED PREVIOUSLY	0-5											0	2			0	2	
	6-10																	
	11-20											0	1			0	1	
	21-30									0	1			0	1	0	2	
	31-40											0	1			0	1	
	41-50											0	1			0	1	
	51-60																	
	61-70																	
TOTALS										0	1	0	5	0	1	0	7	100

R —Recovered D —Died M —Mortality percentage

TABLE LIII

Mortality of cases according to age and sex.

Darrang tea estates, 'B' group

Groups according to treatment of cases	Age groups	MALE			FEMALE			TOTALS		
		Recovered	Died	Mortality	Recovered	Died	Mortality	Recovered	Died	Mortality
NO TREATMENT	0-5		3						3	
	6-10									
	11-20		2		3			5		
	21-30				1			1		
	31-40				2			2		
	41-50									
	51-60									
	61-70									
	TOTALS		5	100	6	100		11	100	
HYPERTONIC SALINE AND PERMANGANATE TREATMENT.	0-5	1	1		1			2	1	
	6-10		2		2				4	
	11-20	1			3	1		4	1	
	21-30	2	4		1			2	5	
	31-40	1			2	2		3	2	
	41-50	2	2		2			2	4	
	51-60									
	61-70									
	TOTALS	7	9	56	6	8	57	13	17	56.6

TABLE LIII—concl'd

Groups according to treatment of cases	Age groups	MALE			FEMALE			TOTALS		
		Recovered	Died	Mortality	Recovered	Died	Mortality	Recovered	Died	Mortality
HYPERTONIC SALINE AND POTASSIUM BICHROMATE TREATMENT, VACCINATED PREVIOUSLY	0-5	2	1		1	1		3	2	
	6-10				1			1		
	11-20				1			1		
	21-30	1				1		1	1	
	31-40	1	3			1		1	4	
	41-50		1		2			2	1	
	51-60									
	61-70									
	TOTALS	4	5	53.5	5	3	37.5	9	8	47.05
VACCINATED PREVIOUSLY	0-5					2			2	
	6-10									
	11-20		1						1	
	21-30					2			2	
	31-40		1						1	
	41-50		1						1	
	51-60									
	61-70									
	TOTALS		3	100		4	100		7	100

TABLE LIV
Infectivity for successive weeks during the epidemic and for different house populations
 Sibsaagar

Groups according to treatment of primary case	Primary cases in house	Population of house	July 10 to 25			July 26 to Aug 1			Aug 2 to 8			Aug 9 to 15			Aug 16 to 22			Aug 23 to Sept 10			Totals			Value of C (Index of infectivity)
			a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	
		1							1	0	0										1	0	0	0
		2							3	3	0										9	9	0	0
		3						1	4	8	1	3	6	0							10	20	2	2
		4						2	4	12	2	9	27	1				1	3	0	21	63	6	6
		5						4	16	64	9	7	28	1				1	4	0	35	140	14	14
		6						10	15	75	15	12	60	5							44	220	35	265
		7						6	16	96	13	13	78	11				2	12	0	42	252	33	224
		8						0	11	77	11	1	7	0							15	105	16	267
		9						6	4	32	2	1	8	0							14	112	9	143
		10						1	7	63	4	3	27	0							17	153	15	176
		11						1	5	50	4	1	10	0							8	80	10	227
		12						1	5	55	6	1	11	0				1	11	0	12	132	7	97
	1	13						5	3	36	6	1	12	1							8	96	13	227
		14							1	13	2	1	13	0							2	26	2	2
		15							1	14	2										1	14	2	2
		16						0				1	15	2							3	45	2	2
		17						6	1	16											2	32	12	12

NO TREAT-

No TREAT- <
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a — Primary cases *b* — Population at risk *y* — Secondary cases

TABLE LIV—*contd*

Groups according to treatment of primary case	Primary cases in house	Population of house	July 19 to 25			July 26 to Aug 1			Aug 2 to 8			Aug 9 to 15			Aug 16 to 22			Aug 23 to Sept 10			TOTALS			Value of <i>C</i> (Index of infectivity)
			<i>a</i>	<i>b</i>	<i>y</i>	<i>a</i>	<i>b</i>	<i>y</i>	<i>a</i>	<i>b</i>	<i>y</i>	<i>a</i>	<i>b</i>	<i>y</i>	<i>a</i>	<i>b</i>	<i>y</i>	<i>a</i>	<i>b</i>	<i>y</i>	<i>a</i>	<i>b</i>	<i>y</i>	
		1							4	8	0	1	2	0	1	1	0	1	1	0	1	1	0	0
		2																			6	12	0	
		3																			3	9	0	
		4																						
		5							4	16	1	1	4	0				2	8	0	7	28	1	57
		6							6	30	0							1	5	0	9	45	0	
		7							9	54	4	4	24	0	1	6	0				14	84	4	82
		8							3	21	0	1	7	1	1	7	0				7	49	3	
		9																						107
		10							1	9	1	2	18	0							4	36	1	
		11							1	10	0										1	10	0	39
		12																			3	33	1	
		13							3	36	2										3	36	2	58
		14																			2	26	0	
		TOTALS							31	184	8	15	107	2	4	20	0	5	17	0	60	369	12	56
		Value of <i>C</i>																						

WITHIN 48 HOURS

[illegible]

TABLE LIV—*concl'd*

Groups according to treatment of primary case	Primary cases in house	Population of house	July 19 to 25			July 26 to Aug 1			Aug 2 to 8			Aug 9 to 15			Aug 16 to 22			Aug 23 to Sept 10			TOTALS			Value of C (Index of infectivity)	
			a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	a	b	y		
BACTERIOPHAGE AFTER 48 HOURS (18 of 29 secondary cases occurred before primary case received bacteriophage)	1	2							1	2	0	1	2	0							2	4	0	93	
		3											1	3	0						3	9	0		
		4											2	8	1						5	20	2		
		5								2	8	1	2	8	1							5	25	0	177
		6							3	15	0	1	5	0	1	5	0					5	25	0	
		7																				7	42	7	265
		8							7	42	7											4	28	5	
		9							1	7	2	1	7	0								4	32	4	175
		10	1	8	2	1	8	1	1	8	0											2	18	1	
		12	1	9	1																	2	22	3	
		13	1	11	2	1	11	1														1	12	1	85
		16																				3	45	1	
		17	1	16	2				2	30	1											1	16	2	
		21							1	15	0											1	20	3	
		24																				1	23	0	
		32							1	31	0											1	31	0	140
		TOTALS	4	44	7	14	121	11	12	113	7	7	37	2	2	13	1	3	19	18	42	347	29		
Value of C				197			112			91			108			149									

TABLE LV
Infectivity for successive weeks during the epidemic and for different house populations.
 Darrang villages.

Groups according to treatment of primary case	Primary cases in house	Population of house	Oct 15 to Nov 11			Nov 12 to 18			Nov 19 to 25			Nov 26 to Dec 2			Dec 3 to 9			Dec 10 to 16			Dec 17 to Jan 6			Totals			Value of C (Index of infectivity)
			a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	
No treatment	1	1																								0	
		2	1	1	0	1	1	0	2	2	0	3	3	0	1	1	0	1	1	0	1	1	0	10	10	0	0
		3				3	6	1	4	8	1	3	6	1	3	6	0	1	2	0	1	2	0	15	30	7	156
		4				3	9	4	6	18	2	2	6	1	3	9	1	4	12	0	1	3	0	19	37	8	156
		5				1	4	0	2	8	0	1	4	1				1	1	0	1	1	0	6	24	1	185
		6							5	25	6	1	5	1	2	10	0	1	20	2	1	5	0	13	65	9	185
		7	1	6	1							1	6	0	1	6	0	1	6	0				1	24	1	118
		8				1	7	0	3	21	3	1	7	1	2	11	3	2	14	0	1	7	0	10	70	7	118
		9							1	8	0													2	16	0	118
		10	2	18	1	2	18	3	1	9	4	18	5	1	1	9	7							8	72	29	310
		11							1	10	0													1	10	0	310
		12				1	11	0																1	11	0	310
		21													1	20	2							1	20	2	310
Totals			4	25	2	12	56	8	24	99	16	15	65	10	16	75	13	16	67	2	6	22	0	93	109	51	203
Value of C			206			260			250			285			36									203			
2						2	3	0	2	4	1	6	13	3							2	5	1	12	25	5	270

TABLE LV—*contd*

Groups according to treatment of primary case	Primary cases in house	Population of house	Oct 16 to Nov 11		Nov 12 to 18		Nov 19 to 25		Nov 26 to Dec 2		Dec 3 to 9		Dec 10 to 16		Dec 17 to Jan 6		Totals		Value of <i>O</i> (index of infectivity)
			<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	
BACTERIOPHAGE WITHIN 48 HOURS, VACCINATED PREVIOUSLY	1	1							2	2	0	1	1	0			3	3	0
		2											1	2	0		1	2	0
		3																	
		4											2	6	0		2	6	0
		5									1	4	0	2	8	0	3	12	0
		6						1	5	0			1	5	0	1	5	0	0
		7											1	6	1		1	6	1
		8									1	7	1				1	7	1
		Totals						3	7	0	3	12	1	7	27	1	14	51	2
		Value of <i>O</i>																62	
	2	6											2	4	0		2	1	0

BACTERIOPHAGE WITHIN 48 HOURS, VACCINATED PREVIOUSLY

TABLE LV—*concd*

Groups according to treatment of primary case	Primary cases in house	Population of house	Oct 16 to Nov 11			Nov 12 to 18			Nov 19 to 25			Nov 26 to Dec 2			Dec 3 to 9			Dec 10 to 16			Dec 17 to Jan 6			TOTALS			Value of C (Index of infectivity)
			a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	
VACCINATED ONLY	1	2										1	1	0				1	1	0				2	2	0	0
		3										2	4	0				1	2	0				3	6	0	0
		4																1	3	0				1	3	0	0
		6																					1	5	0	0	
		12											1	11	0									1	11	0	0
		TOTALS				1	5	0				4	16	0				3	6	0				8	27	0	0

[illegible]

No 36 rejected no record of population

a—Primary cases *b*—Population at risk

Secondary cases

TABLE LVI

Infectivity for successive weeks during the epidemic and for different house populations.

Darrang tea estates, 'A' group

Groups according to treatment of primary case	Primary cases in house	Population of house	Sept 13 to 29			Sept 30 to Oct 6			Oct 7 to 13			Oct 14 to 26			Totals			Value of C (Index of infectivity)
			a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	
NO TREATMENT	1	1				1	0	0							1	0	0	320
		2				1	1	0							1	1	0	
		3				1	2	1	1						2	4	1	
		5				1	5	1	2	8					2	8	2	
		6				1									1	5	1	
		TOTALS				4	8	2	3	10	2				7	19	4	320
		Value of C				320									320			
VACCINATED ONLY	2	6							2	4	1				2	4	1	333
		3				1	2	0	2	4	1	2	4	1	5	10	2	148
		4							1	3	0	2	6	0	3	9	0	
		TOTALS				1	2	0	3	7	1	4	10	1	8	19	2	148
		Value of C				154						143			148			

	PERMANGANATE AND HYPERTONIC SALINE										
	1	2	3	4	5	6	7	8	9	10	
1	1	1	0	0	0	0	1	0	0	0	0
	2	1	0	0	0	0	1	0	0	0	0
	3	1	0	0	0	0	1	0	0	0	0
	4	1	0	0	0	0	1	0	0	0	0
	5	1	0	0	0	0	1	0	0	0	0
	6	1	0	0	0	0	1	0	0	0	0
	7	1	0	0	0	0	1	0	0	0	0
	8	1	0	0	0	0	1	0	0	0	0
	9	1	0	0	0	0	1	0	0	0	0
	10	1	0	0	0	0	1	0	0	0	0
Totals	2	1	0	0	0	0	1	0	0	0	0
Value of C	2	1	0	0	0	0	1	0	0	0	0
2	1	2	2	2	2	2	3	3	3	3	3
1	1	1	1	1	1	1	1	1	1	1	1
	2	1	1	1	1	1	1	1	1	1	1
	3	1	1	1	1	1	1	1	1	1	1
	4	1	1	1	1	1	1	1	1	1	1
	5	1	1	1	1	1	1	1	1	1	1
	6	1	1	1	1	1	1	1	1	1	1
	7	1	1	1	1	1	1	1	1	1	1
	8	1	1	1	1	1	1	1	1	1	1
	9	1	1	1	1	1	1	1	1	1	1
	10	1	1	1	1	1	1	1	1	1	1
Totals	1	2	2	2	2	2	3	3	3	3	3
Value of C	1	2	2	2	2	2	3	3	3	3	3
1	1	1	1	1	1	1	1	1	1	1	1
	2	1	1	1	1	1	1	1	1	1	1
	3	1	1	1	1	1	1	1	1	1	1
	4	1	1	1	1	1	1	1	1	1	1
	5	1	1	1	1	1	1	1	1	1	1
	6	1	1	1	1	1	1	1	1	1	1
	7	1	1	1	1	1	1	1	1	1	1
	8	1	1	1	1	1	1	1	1	1	1
	9	1	1	1	1	1	1	1	1	1	1
	10	1	1	1	1	1	1	1	1	1	1
Totals	1	2	2	2	2	2	3	3	3	3	3
Value of C	1	2	2	2	2	2	3	3	3	3	3
1	1	1	1	1	1	1	1	1	1	1	1
	2	1	1	1	1	1	1	1	1	1	1
	3	1	1	1	1	1	1	1	1	1	1
	4	1	1	1	1	1	1	1	1	1	1
	5	1	1	1	1	1	1	1	1	1	1
	6	1	1	1	1	1	1	1	1	1	1
	7	1	1	1	1	1	1	1	1	1	1
	8	1	1	1	1	1	1	1	1	1	1
	9	1	1	1	1	1	1	1	1	1	1
	10	1	1	1	1	1	1	1	1	1	1
Totals	1	2	2	2	2	2	3	3	3	3	3
Value of C	1	2	2	2	2	2	3	3	3	3	3

a — Primary cases *b* — Population at risk *y* — Secondary cases

TABLE LVII

Infectivity for successive weeks during the epidemic and for different house populations

Darrang tea estates, 'B' group

Groups according to treatment of primary case	Primary cases in house	Population of house	Nov 26 to Dec 2			Dec 3 to 9			Dec 10 to 16			Dec 17 and after			Totals			Value of C (Index of infectivity)
			a	b	y	a	b	y	a	b	y	a	b	y	a	b	y	
No TREATMENT	1	1										1	0	0	1	0	0	98
		3										3	6	1	3	6	1	
		5										1	4	0	1	4	0	
		6										2		0	2		0	
		7				1	6	0		10	0				2	10		
		TOTALS				1	6	0		3	16	1	5	10	9	32	2	98
AND HYPER- TONIC VED TO HOSPITAL	1	Value of C				77			133			98						73
		6				2	4	1							2	4	1	
		1				2						1	0	0	1	0	0	
		2	1			1	2	0		1	4				3	3	0	
		3	2			2	2			2	6	1	3	0	5	6	2	
	2	4								12					3	15	0	333
		5								20					5	12	2	
		6								4	20				3	20	0	
		7								12		2	12	0	4	24	0	
		8								8		1	7	0	4	7	0	
		9							1			1		0	1	9	0	

PERMANGANATE AND HYPERTONIC SALINE, VACCINATED PREVIOUSLY	1	{	TOTALS			5	10	2	15	63	0	5	22	0	25	93	2	13		
			Value of C	0																
			3														2	1	0	0
			1														1	0	0	0
			1	1	2	0	1	0	0								1	0	0	0
			3														3	0	0	0
			4														3	0	0	0
			5														1	4	1	5
			7														2	14	0	0
			TOTALS														10	33	6	279
Value of C	64																			
PERMANGANATE PREVIOUSLY VACCINATED	1	{	TOTALS			2	7	5	3	20	1	2	1	0	10	33	6	279		
			Value of C	933																
			3														2	1	0	0
			1														1	0	0	0
			1	1	2	0	1	0	0								1	0	0	0
			3														1	0	0	0
			4														1	0	0	0
			5														1	0	0	0
			7														1	0	0	0
			TOTALS														4	15	2	210
Value of C	210																			
VACCINATED PREVIOUSLY	1	{	TOTALS												1	15	2	210		
			Value of C	64																
			3														2	1	0	0
			1														1	0	0	0
			1	1	2	0	1	0	0								1	0	0	0
			3														1	0	0	0
			4														1	0	0	0
			5														1	0	0	0
			7														1	0	0	0
			TOTALS														4	15	2	210
Value of C	210																			

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a — Primary cases b — Population at risk η — Secondary cases

TABLE LVII

Infectivity for successive weeks during the epidemic and for different house populations

Darrang tea estates, 'B' group

Groups according to treatment of primary case	Primary cases in house	Population of house	Nov 26 to Dec 2			Dec 3 to 9			Dec 10 to 16			Dec 17 and after			Totals			Value of C (Index of infectivity)		
			a	b	y	a	b	y	a	b	y	a	b	y	a	b	y			
NO TREATMENT	1	1										1	0	0	1	0	0	98		
		3										3	6	1	3	6	1			
		5										1	4	0	1	4	0			
		6													2	10	0			
		7				1	6	0	2	1	1				2	12	1			
		Totals			1	6	0	3	16	1	5	10	1	9	32	2				
		Value of C			77						133						98			
	2	6				2	4	1							2	4	1	333		
		Value of C			77						133						98			
		Value of C			77						133						98			
AND HYPERTONIC VLD TO HOSPITAL	1	1										1	0	0	1	0	0	93		
		2													3	3	0			
		3													3	6	0			
		4													5	15	2			
		5													3	12	0			
		6													4	20	0			
		7													4	24	0			
		8													4	7	0			
		9													1	8	0			
		Totals			2	1	2	2	6	2	1	1	2	7	1	1	1		1	

A PRELIMINARY NOTE ON THE ELECTRICAL CHARGE CARRIED BY THE RABIES VIRUS

BY

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THE present note records the preliminary observations made in the course of work, initiated by Sir Robert McCarrison, on the behaviour of the virus of rabies in an electric field. The procedure adopted was cataphoresis of emulsions of rabies-infected brain.

Filtration experiments

The first step in our investigation was to carry out certain filtration experiments in order to learn what medium to employ in the preparation of the emulsions of the brain of rabid animals that were needed for our purpose, and, further, what should be the concentration of brain tissue in these emulsions. The results of these experiments are given in Table I.

It was apparent from the results set out in Table I that serum was the best medium to employ. The rabies virus migrated more rapidly into the fluid medium when serum was used than when either saline or broth was used. Indeed, the experiments provide no evidence that the virus migrated either into saline or broth. Our experiments indicated, also, that a five per cent emulsion in sheep's serum gave the best results.

II	0-7-33	8 per cent	Nutrient broth	do	7-7-33	12	Nil
	0-7-33	8 per cent	Sheep serum	do	7-7-33	13	Nil
						14	Rabies developed on 8th day
	14-7-33	5 per cent	Saline	Gauro and I B candle	15-7-33	15	Rabies developed on 7th day
III	11-7-33	5 per cent	Nutrient broth	do	15-7-33	16	Nil
	14-7-33	5 per cent	Sheep serum	do	15-7-33	17	Nil
						18	Nil
	14-7-33	5 per cent	Sheep serum	do	15-7-33	19	Nil
IV	24-8-33	5 per cent	Sheep serum	do	25-8-33	20	Rabies developed on 8th day
	24-8-33	5 per cent	Sheep serum	do	25-8-33	21	Rabies developed on 8th day
						22	Rabies developed on 7th day
	24-8-33	5 per cent	Sheep serum	do	25-8-33	23	Rabies developed on 8th day

Material

A five per cent emulsion of the brain of rabbits, showing typical signs of rabies, was made up in sterile sheep's serum. Such emulsions were used throughout our cataphoresis experiments. About 25 c c of the emulsion were used for each experiment. We have performed the cataphoresis with the fixed virus (Paris) strain, kept in the Pasteur Institute, Coonoor, by passages through healthy rabbits. The buffer used in all experiments was Sorensen's phosphates at pH 7.38.

Technique.

Not having Todd's, nor any other ready-made, apparatus in this Laboratory a simple cell (Fig. 1) was devised by one of us (G. S.) for cataphoresis experiments. The cell is made of $\frac{1}{4}$ -inch glass tubing. Its main stem is approximately 6 inches in length and is constricted slightly at either end. Attached to its upper part, about two-and-a-half inches from the top, is a narrow bent arm of $\frac{1}{4}$ -inch glass tubing.

Two such cells are needed to make the two poles of the current. The two cells are thoroughly cleaned with chromic acid and washed several times before use, first with tap and then with distilled water. They are then wrapped in paper and sterilized, preferably in an autoclave. After sterilization, a glass tap is fitted to the top (A, Fig. 2) of each cell by means of rubber tubing. By sucking through this tap, while keeping the narrow side-arm (C, Fig. 2) closed, a hot 2 per cent agar solution, made up in 0.9 per cent saline, is introduced into the cell and allowed to form a gel. The agar, which is capable of conducting an electric current, forms a seal for the lower end (B, Fig. 2) of the cell. After sealing the lower end in this way, the end of the narrow side-arm (C, Fig. 2) is dipped into the required sterile buffer solution which is then sucked into the cell in amount sufficient to fill it. The tap at the top of the cell is then closed, the buffer solution now remains in position when the cell is held vertically. During an actual cataphoresis experiment the narrow side-arms of the two cells, filled with buffer, are lowered vertically into a sterile beaker (1, Fig. 2) containing the requisite suspension of the virus, the mouth of this beaker being protected from bacterial contamination by means of a cap. The agar gel ends (B, Fig. 2) of the two cells are immersed in two beakers (2, Fig. 2) containing a 10 per cent solution of sodium chloride. These two beakers are connected, by means of two U-shaped agar bridges (4, Fig. 2) of about 1.5 mm bore, to two other beakers (3, Fig. 2) containing 10 per cent copper sulphate solution. Two stout copper terminals, from the source of the electric current, are then dipped into the beakers containing the copper sulphate solution. One forms the positive and the other the negative pole, the cells adjacent to each being respectively the 'positive' and the 'negative' cell. It is advisable to introduce into the circuit either a milliammeter or a galvanometer with which to measure the amount of current passing through the circuit. Several resistances of the order of ten to fifty thousand ohms for a voltage of 220 may be needed to reduce the current down to about 4 milliamperes, which we have found to be optimal for our work. It is essential to use a current of this low amperage in order to avoid undue electrolysis and heating. Fig. 2 gives the general lay-out of the apparatus.

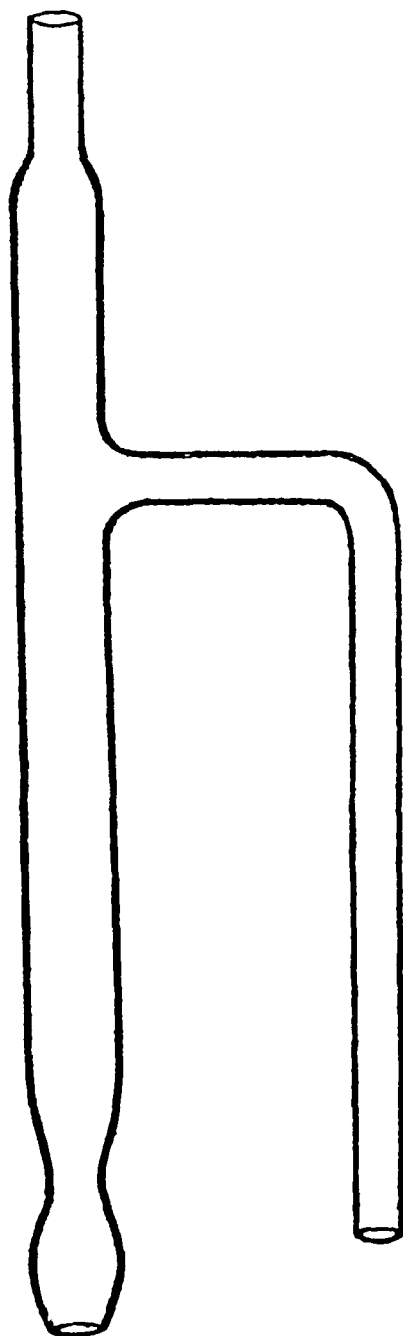


Fig 1 —The cataphoric cell actual size

Within 30 minutes of the passage of the current a faint turbidity begins to appear and to rise up the side-arm (C, Fig 2) of the cell attached to the positive pole, while the contents of that attached to the negative pole remain clear. With the continued passage of the current for the required period—a period determined by the amount of material migrating into the cells—the turbidity in the positive cell rises higher and higher, while the contents of the negative cell continue to remain clear. In the present experiments the current was run continuously from $2\frac{1}{2}$ to $4\frac{1}{2}$ hours by which time enough material had collected for inoculation purposes.

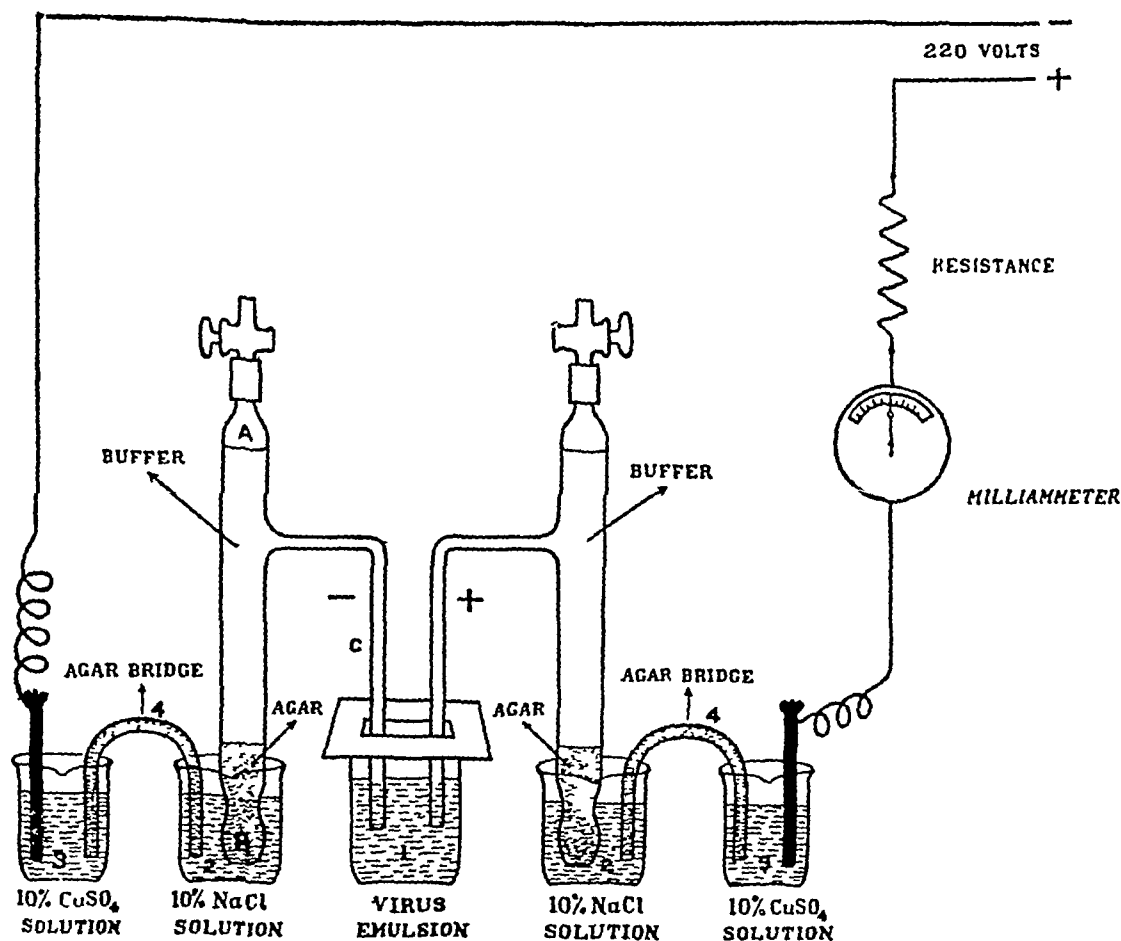


Fig 2—Showing the general lay out of the cataphoresis apparatus. For description see text.

At the end of the required period the current is not immediately stopped, the two cells being first removed in order to prevent a reverse migration of virus particles due to any reverse current that might be set up by the charged particles in each cell. It is to be emphasized that the current must not be stopped until the cells have been removed. After their removal the ends of the side-arms (C, Fig 2) that have been in contact with the brain emulsion are first thoroughly wiped with sterile gauze and then a few drops of the fluid contained in the side-arms are run out from each, this is effected by opening the glass tap at the top of each cell. In this way

TABLE II

Young details of six experiments wherein materials, derived by cataphoresis of a 5 per cent emulsion of rabid [*fixed virus* (Paris)] rabbit's brain in sheep's serum, were inoculated subcutaneously into rabbits, in doses of 0.2 c.c.

Number of experiment	PARTICULARS OF CATAPHORESIS		Duration of passage of current (hours)	CHANGES IN CONSTANTS OF		INOCULATION WITH MATERIAL FROM NEGATIVE CELL		INOCULATION WITH MATERIAL FROM POSITIVE CELL	
	Voltage	Amperage		Negative cell	Positive cell	Serial number of rabbit	Results	Serial number of rabbit	Results
V	220	About 1 milliamperes	1	Clear	Turbid	{ 24 25	No rabies do	26 27	Rabies developed on 7th day Rabies developed on 7th day
VI	do	do	1	do	do	{ 28 29	do do	30 31	Rabies developed on 7th day Rabies developed on 7th day
VII	do	do	1½	do	do	{ 32 33	do do	34 35	Rabies developed on 7th day Rabies developed on 7th day
VIII	do	do	4½	do	do	{ 36 37	do do	38 39	Rabies developed on 7th day Rabies developed on 9th day
IX	do	do	3½	do	Rapidly turbid	{ 40 41	do do	42 43	Rabies developed on 7th day Rabies developed on 7th day
X	do	do	2½	do	do	{ 44 45	do do	46 47	Rabies developed on 7th day Rabies developed on 7th day

of the same concentration (5 per cent) The same apparatus was used, the same observer (G S) conducted the electrophoresis, the current being passed through the emulsions for approximately the same length of time (4 to 5 hours) The material accumulating in the positive cell and that contained in the negative cell of the apparatus, following electrophoresis, was collected in the same way and used for inoculation purposes The same observer (W A B) carried out the inoculations in the presence of the others, the routine procedure of this Institute for the subdural inoculation of passage animals was followed Rabbits, sheep and guinea-pigs were used for experimental purposes Ether anaesthesia was employed The inoculated animals were kept under observation for the same length of time The dose of the inoculated material was 0.5 c.c. for sheep and 0.2 c.c. for rabbits and guinea-pigs

Experiments

Full details of the experiments—22 in number—are given in Table I

(1) *Electrophoresis of emulsions in sheep's serum of normal rabbit's brain*

In scrutinizing the observations recorded in the preliminary note, the question arose as to whether material derived by electrophoresis from apparently normal brain, or from the sheep's serum in which the emulsions were made, might not be capable of causing symptoms resembling rabies when inoculated subdurally into rabbits The necessity to exclude any such possibility is made evident by the recent observation of Friedemann and Elkeles (1933) that the tissues of apparently normal rabbits may contain a living agent, possibly of virus nature, capable of causing encephalitis on subdural inoculation into rabbits

A five per cent emulsion of normal rabbit brain was made up in sheep's serum and subjected to electrophoresis in the usual way (experiment 11, Table I) Material from the negative cell and material from the positive cell of the apparatus was inoculated subdurally into rabbits in doses of 0.2 c.c. The results were negative neither that from the negative nor that from the positive cell caused any untoward symptoms in the inoculated animals It was not considered necessary to repeat this experiment since it demonstrated sufficiently clearly that *normal* brain was harmless What symptoms might be produced by the subdural inoculation of material derived by electrophoresis from brain that is abnormal, from causes other than rabies, is another matter

The results of this experiment also excluded the possibility that the sheep's serum, used in the preparation of the brain emulsion, might yield substances, as a result of electrophoresis, capable of causing encephalitic symptoms on subdural inoculation into rabbits Nevertheless, another experiment (No 12, Table I) was carried out in which sheep's serum, without added brain substance, was subjected to electrophoresis The materials contained in the positive and in the negative cells of the apparatus were inoculated subdurally into rabbits The results were negative It was concluded, therefore, that whatever caused the rabies-like symptoms in the animals referred to in the preliminary note, it had nothing to

do either with *normal* brain tissue or with *normal* sheep's serum but was peculiar to rabies infected brain tissue

(2) *Experiments with street virus*

The brain of a supposedly rabid dog, received at this Institute for report, was emulsified and inoculated subdurally into a guinea pig in the customary way. It developed rabies. The brain of this guinea-pig was taken and a 5 per cent emulsion in sheep's serum was prepared from it. This emulsion was subjected to electrophoresis for 5 hours. The material that had accumulated in the positive cell of the apparatus ('positive material') was then withdrawn with the precautions described in the preceding note and a dose of 0.2 c.c. was inoculated subdurally into each of two guinea-pigs. Similarly, that contained in the negative cell ('negative material') was withdrawn and a dose of 0.2 c.c. inoculated subdurally into each of two guinea-pigs. Both animals inoculated with the 'positive material' developed rabies on the eleventh and twelfth day, respectively, following inoculation, and Negri bodies were present in the hippocampus of both. Neither of the guinea-pigs inoculated with the 'negative material' developed rabies (experiment 13, Table I).

In a second experiment with street virus the same procedure was followed. In this, also both guinea-pigs inoculated subdurally with the 'positive material' developed rabies on the thirteenth and sixteenth day respectively, and Negri bodies were present in the hippocampus of both. The two guinea-pigs that received subdural inoculations of the 'negative material' died, the first on the fifteenth day following inoculation and the second on the sixtieth day. In neither case were Negri bodies present, showing that their death was not due to rabies (experiment 14, Table I). These experiments are of special importance since, apart from the clinical evidence of rabies, the presence of Negri bodies in the hippocampus of the four guinea-pigs, inoculated with the 'positive material' demonstrates conclusively that the disease produced by this material was actually rabies and not a malady having a close clinical resemblance to it.

(3) *Experiments with fixed virus (Paris)*

Experiments (Nos 12 and 13, Table I) with street virus had made it clear that the disease caused by the inoculation of our 'positive material' was actually rabies, the diagnosis having been confirmed by the demonstration of Negri bodies in the hippocampus of the animals inoculated with this material. But where we are concerned with fixed virus such demonstration of Negri bodies is rarely or never possible. 'A peculiar and most important difference between natural rabies and the rabies which has passed through hundreds of rabbits subdurally, and is known as the fixed virus of antirabic institutes, is the fact that whereas Negri bodies are abundant and easily recognized in the former, in the latter they are either absent or so small as to be seen with difficulty' (Cornwall, 1908). The material which we have used in the experiments now to be dealt with was fixed virus (Paris) which had been passed through thousands of rabbits. In order, therefore, to demonstrate that the disease caused by our 'positive material', derived by electrophoresis from brains infected with fixed virus, was really rabies we had to rely on clinical signs and symptoms.

TABLE

Giving details and results

Number of experiment	Date of experiment	Material used	How material was treated	CHANGES IN CONTENTS OF		INOCULATION OF MATERIAL FROM NEGATIVE CELL INTO		RESULTS OF INOCULATION WITH MATERIAL FROM NEGATIVE CELL	
				Negative cell	Positive cell	Rabbit or G pig number	Sheep number	Rabbit or G pig	Sheep
11	14-12-33	Normal rabbit's brain	Electrophoresis	Clear	Opalescent	48		Nil	
		do	do	do	do	49		Nil	
12	25-1-34	Normal sheep's serum without added brain tissue	Kept in cold storage for about 30 days						
		do	Electrophoresis			138		Nil	
		do	do			139		Nil	
13	9-11-33	Guinea pig brain, street virus, first passage from rabid dog's brain	do	Clear	Turbid	G p 1		Nil	
		do	do			G p 2		Nil	
14	14-11-33	Guinea pig brain, street virus	do	Clear	Turbid	G p 5		Died	
		do	do			G p 6		do	
		do	do						
		do	do		..				
15	18-12-33	Brain Pasteur Institute passage rabbit F V Paris	do	Clear	Turbid	52		Nil	
		do	do	do	do	53		Nil	
16	29-12-33	Brain of rabbit No 54	Nil						
		do	Nil						

of the experiments

INOCULATION OF MATERIAL FROM POSITIVE CELL INTO		RESULTS OF INOCULATION WITH MATERIAL FROM POSITIVE CELL		INOCULATION OF ORIGINAL BRAIN EMULSION INTO		RESULTS OF INOCULATION WITH ORIGINAL BRAIN EMULSION		REMARKS
Rabbit or pig number	Sheep number	Rabbit or G p g	Sheep	Rabbit number	Sheep number	Rabbit	Sheep	
50		Nd						
51		Nd						
140		Nd						
141		Nd						
G p 3		+						Subdural inoculation made into two rabbits (Nos 136 and 137) no effect
G p 4		+						G p 3 developed rabies on the 11th day Negri bodies present in hippocampus
								G p 4 developed rabies on the 12th day Negri bodies present in hippocampus
								G p 5 died on the 15th day no Negri bodies present in hippocampus
								G p 6 died on the 60th day no Negri bodies present in hippocampus
G p 7		+						G p 7 developed rabies on the 13th day Negri bodies present in hippocampus
G p 8		+						G p 8 developed rabies on the 16th day Negri bodies present in hippocampus
54		+						No 54 developed rabies on the 8th day
55		+						No 55 developed rabies on the 9th day
				56		+		No 56 developed rabies on the 7th day
				57		+		No 57 developed rabies on the 8th day

TABLE

Number of experiment	Date of experiment	Material used	How material was treated	CHANGES IN CONTENTS OF		INOCULATION OF MATERIAL FROM NEGATIVE CELL INTO		RESULTS OF INOCULATION WITH MATERIAL FROM NEGATIVE CELL	
				Negative cell	Positive cell	Rabbit or G pig number	Sheep number	Rabbit or G pig	Sheep
17	21-12-33	Brain, Pasteur Institute passage rabbit, F V Paris	Electrophoresis	Clear	Turbid	58		Died	
		do	do	do	do	59		Nil	
18	29-12-33	Brain of rabbit No 60	Nil						
		do	Nil						
19	19-12-33	Brain, Pasteur Institute passage rabbit, F V Paris	Electrophoresis	Clear	Turbid		1		Nil
20	20-12-33	Brain, Pasteur Institute passage rabbit, F V Paris	do	do	do		3		Nil
21	2-1-34	Brain of Sheep No 4	Nil						
		do	Nil						
		do	Electrophoresis	Clear	Turbid	64	5	Nil	Nil
		do	do	do	do	65		Nil	
22	5-1-34	I Hippocampus of rabbit No 57	Nil						
		do	Nil						
		do	Electrophoresis	Clear	Turbid	72		Nil	
		do	do	do	do	73		Nil	
23	6-1-34	II Hippocampus of rabbit No 56	Nil						
		do	Nil						
		do	Electrophoresis	Clear	Turbid	78		Nil	
		do	do	do	do	79		Nil	

I—contd

INOCULATION OF MATERIAL FROM POSITIVE CELL INTO		RESULTS OF INOCULATION WITH MATERIAL FROM POSITIVE CELL		INOCULATION OF ORIGINAL BRAIN EMULSION INTO		RESULTS OF INOCULATION WITH ORIGINAL BRAIN EMULSION		REMARKS
Rabbit or G pig number	Sheep number	Rabbit or G pig	Sheep	Rabbit number	Sheep number	Rabbit	Sheep	
60		+						No 58 died on 6-1-34 brain negative to rabies, by inoculation into another rabbit
61		+						Nos 60 and 61 developed rabies on the 7th day
				62		+		No 62 developed rabies on the 7th day
				63		+		No 63 developed rabies on the 7th day
	2		Nil					Sheep No 2 did not develop rabies
	4		+					Sheep No 4 developed rabies on the 12th day
				68	7	+	+	Nos 68 and 69 developed rabies on the 7th day
				69		+		Sheep No 7 developed rabies on the 7th day
66	6	+	+					No 66 developed rabies on the 7th day No 67 on the 7th day
67		+						Sheep No 6 developed rabies on the 7th day
				70		+		No 70 developed rabies on the 7th day
				71		+		No 71 developed rabies on the 7th day
74		+						No 74 developed rabies on the 7th day
75		+						No 75 developed rabies on the 7th day
				76		+		No 76 developed rabies on the 7th day
				77		+		No 77 developed rabies on the 7th day
80		+						No 80 developed rabies on the 7th day
81		+						No 81 developed rabies on the 7th day

Series of ampoules (I, II, III and IV) sent to Colonel Shortt

TABLE

Number of experiment	Date of experiment	Material used	How material was treated	CHANGES IN CONTENTS OF		INOCULATION OF MATERIAL FROM NEGATIVE CELL INTO		RESULTS OF INOCULATION WITH MATERIAL FROM NEGATIVE CELL	
				Negative cell	Positive cell	Rabbit or G pig number	Sheep number	Rabbit or G pig	Sheep
29	14-1-34	Ampoule No 6 from experiment 23 of 6-1-34 original brain emulsion	Kept at room temperature from 6-1-34 to 14-1-34						
		Ampoule No 4, from experiment 23 of 6-1-34 from negative cell	do			114		<i>Nil</i>	
		Ampoule No 5 from experiment 23 of 6-1-34 from positive cell	do			115		<i>Nil</i>	
30	14-1-34	Ampoule No 9, from experiment 24 of 7-1-34 original brain emulsion	Kept at room temperature from 7-1-34 to 14-1-34						
		Ampoule No 7, from experiment 24 of 7-1-34 from negative cell	do			120		<i>Nil</i>	
		Ampoule No 8, from experiment 24 of 7-1-34 from positive cell	do			121		<i>Nil</i>	
31	16-1-34	Ampoule No 12 from experiment 25 of 8-1-34 original brain emulsion	Kept at room temperature from 8-1-34 to 16-1-34						
		Ampoule No 10 from experiment 25 of 8-1-34 from negative cell	do			126		<i>Nil</i>	
		Ampoule No 11 from experiment 25 of 8-1-34 from positive cell	do			127		<i>Nil</i>	
32	15-1-34	Emulsion of brain of Pasteur Institute passage rabbit, F V Paris	Kept in cold storage for 48 hours						
		do	Electrophoresis			132		<i>Nil</i>	
			do			133		<i>Nil</i>	

I—concl'd

INOCULATION OF MATERIAL FROM POSITIVE CELL INTO		RESULTS OF INOCULATION WITH MATERIAL FROM POSITIVE CELL		INOCULATION OF ORIGINAL BRAIN EMULSION INTO		RESULTS OF INOCULATION WITH ORIGINAL BRAIN EMULSION		REMARKS
Rabbit or G pig number	Sheep number	Rabbit or G pig	Sheep	Rabbit number	Sheep number	Rabbit	Sheep	
				112		+		No 112 developed rabies on the 8th day
				113		+		No 113 developed rabies on the 7th day
116		+						No 116 developed rabies on the 8th day
117		+						No 117 developed rabies on the 8th day
				118		+		No 118 developed rabies on the 7th day
				119		+		No 119 developed rabies on the 8th day
122		+						No 122 developed rabies on the 7th day
123		Nil						No 123 had marked hemorrhage at operation <i>no rabies</i>
				124		+		No 124 developed rabies on the 7th day
				125		+		No 125 developed rabies on the 8th day
128		+						No 128 developed rabies on the 8th day
129		+						No 129 developed rabies on the 7th day
				130		+		No 130 developed rabies on the 7th day
134		+		131		+		No 131 developed rabies on the 7th day
135		Nil						No 134 developed rabies on the 7th day
								No 135 did not develop rabies

Compare with Colonel Shortt's results

In this Institute the normal incubation period of fixed virus rabies is from 7 to 9 days for rabbits, and from 6 to 12 days for sheep. The train of symptoms, both in rabbits and in sheep, is very familiar to one of us (W. A. B.), who as Assistant to the Director, Pasteur Institute, Coonoor, comes into daily contact with the disease and has inoculated some thousands of animals with fixed virus in the ordinary course of his routine duties. It was thought, therefore, that if the routine method of inoculation followed at this Institute were adopted in our experiments, if the symptoms arising from inoculation of our 'positive material' were clinically indistinguishable from rabies, if they appeared at the normal incubation time, and if the disease produced in this way could be conveyed to rabbits and sheep—the normal incubation period and the character of the symptoms being preserved unaltered—then there could be no reasonable doubt but that they were in fact those of rabies and not those of a condition having a close clinical resemblance to it. The results of 10 experiments (Nos 15, 16, 17, 18, 20, 21, 22, 23, 26 and 27, Table I) make it clear that this was indeed so. With the material accumulating in the positive cell of our apparatus, following electrophoresis of emulsions in sheep's serum of the brains of Pasteur Institute passage rabbits, we have produced a disease clinically indistinguishable from rabies. We have then taken the brains of animals suffering from this disease, prepared emulsions in sheep's serum from them, subjected these emulsions to electrophoresis, and with the material that accumulated in the positive cell have again produced the disease both in rabbits and in sheep. Similarly, we have produced the disease in sheep by means of our 'positive material' and from them conveyed it—again by our 'positive material'—to sheep and to rabbits. In all these experiments the incubation period has been the same as that of true rabies and the symptoms have been those characteristic of this disease.

It will be noted from Table I that there are twelve experiments (Nos 21 to 32) in which the original emulsions of rabies-infected brains were used for subdural inoculation into animals as well as the materials derived from them by electrophoresis. In all of these the results have been the same—

- (i) the original emulsion invariably caused rabies,
- (ii) the material collecting in the positive cell, following electrophoresis of this emulsion, almost invariably caused rabies,
- (iii) the material contained in the negative cell, following electrophoresis of this emulsion, never caused rabies.

Looking through the entire list of experiments (Table I) it will be seen that 28 rabbits and 3 sheep received subdural inoculations of emulsions of rabies-infected brains, they all developed rabies. Twenty-eight rabbits and 5 sheep received subdural inoculations of the material contained in the negative cell following electrophoresis of these emulsions, none of them developed rabies. Twenty-eight rabbits and 5 sheep received subdural inoculations of the material collected in the positive cell following electrophoresis of these emulsions, all developed rabies. There were thus three failures. Of the two failures amongst rabbits, one was due to profuse hæmorrhage into the brain, apparently due to non-infective material. It was not due to non-infective material, for the same material caused rabies in Colonel Shortt's animals (*vide* above).

(experiment 32) due to non-infectivity of the 'positive material', for another rabbit (No 131), inoculated with it at the same time, developed rabies. In both cases, therefore the failure was accidental. Such failures are occasionally met with in the ordinary course of passage inoculation in this Institute. Thus of 2,465 rabbits, inoculated subdurally by one of us (W A B) during the past 9 years, 64 failed to develop rabies. Escapes are usually due to technical slips, such as too rapid withdrawal of the needle or the failure of the assistant to exert top pressure with the cotton wool swab when the operator is injecting the emulsion and withdrawing the needle (Harvey and Acton, 1922). The failure to cause the disease in one of the 5 sheep inoculated with our 'positive material' may have been due to some such technical fault at the time of operation, although none was observed to have occurred, or the animal may have been immune. But whatever the cause, we have no evidence in this case that the 'positive material' used was either infective or non-infective since no other animal was inoculated with it. Of such escapes there have been 17 amongst 761 sheep in this Institute during the past 9 years.

One other experiment (No 32) may be referred to here. It was designed to learn whether keeping an emulsion of rabies-infected brain in cold storage for 48 hours, before it was subjected to electrophoresis, would impair the virulence of the subsequent 'positive' product or impart to it noxious qualities other than its rabies-producing properties. So far as this possibility was concerned the experiment yielded negative results. It provided, however, further evidence of the fact that electrophoresis of rabies-infected brain emulsion in sheep's serum causes negatively charged particles capable of producing rabies to collect in the positive cell of the apparatus.

(4) *Experiments carried out by Colonel Shortt at the Pasteur Institute of India, Kasauli, with material sent from Coonoor*

At this stage of our investigations it was thought well to submit the material derived by electrophoresis from rabies-infected brains (Paris fixed virus) to an independent observer for experimental trials. Four series (I, II, III and IV) of three ampoules (*vide* experiments 22, 23, 24 and 25, Table I) were accordingly sent to Lieut-Col H E Shortt, I M S, Director, Pasteur Institute of India Kasauli, who was kind enough to co-operate with us. The ampoules sent to him were numbered 1 to 12. Each had the dose (0.2 c.c.), for subdural inoculation into rabbits, marked upon it but nothing else. Accompanying each series of 3 ampoules was a sealed envelope containing a key to the nature of their contents, this envelope to be opened by Colonel Shortt on the eighth or ninth day following his inoculation experiments. One ampoule in each series contained the original emulsion of rabies-infected brain, a second the material collected from the positive cell* following electrophoresis of this emulsion, and the third the material contained in the negative cell* after this electrophoresis. Ampoules Nos 1 to 6 contained materials derived from rabbits that had developed rabies as a result of inoculation with our 'positive material'. Ampoules Nos 7 to 12 contained material derived from the usual passage rabies of the Pasteur Institute, Coonoor.

* Referred to in Colonel Shortt's report as 'positive (pos) pole' and 'negative (neg) pole' respectively.

TABLE II
Giving the results of Colonel Shott's inoculation experiments with material sent from Coonoor

Series	Ampoule number	Animal number	Date of subdural inoculation	SYMPTOMS WITH DATES					REMARKS
				T	S	F	L	D	
I	1 Negative pole	1A	12-1-34						Animals alive and well
		1B	12-1-34						
	2 Positive pole	2A	12-1-34	20-1-34	21-1-34	21-1-34	22-1-34	22-1-34	
		2B	12-1-34	21-1-34	21-1-34	22-1-34	23-1-34	23-1-34	
	3 Original brain emulsion	3A	12-1-34	19-1-34	20-1-34	20-1-34	21-1-34	21-1-34	
		3B	12-1-34	19-1-34	20-1-34	20-1-34	21-1-34	22-1-34	
	4 Negative pole	4A	14-1-34						Animals alive and well
		4B	14-1-34						
	5 Positive pole	5A	14-1-34	23-1-34	23-1-34	24-1-34	25-1-34	25-1-34	Died in 24 hours Re done on 16-1-34 on account of accidental death of 5B. Symptoms typical of rabies
		5B	14-1-34					15-1-34	
		5B'	16-1-34	26-1-34	26-1-34	27-1-34	27-1-34		
	6 Original brain emulsion	6A	14-1-34					17-1-34	Both died after 48 hours due to sepsis
		6B	14-1-34					17-1-34	

		6A'	6B'	17-1-34	24-1-34	24-1-34	25-1-34	25-1-34	22-1-34	20-1-34	Died after 5 days of septicaemia and developed rabies in typical fashion. The original material was tested culturally and found to be contaminated
II	7	Negative pole	7A	14-1-34							Animals alive and well
			7B	14-1-34							
III	8	Positive pole	8A	14-1-34	21-1-34	21-1-34	22-1-34	22-1-34	23-1-34	23-1-34	
			8B	14-1-34	21-1-34	21-1-34	22-1-34	22-1-34	23-1-34	23-1-34	
	9	Original brain emulsion	9A	14-1-34	21-1-34	21-1-34	22-1-34	22-1-34	23-1-34	23-1-34	
			9P	14-1-34	21-1-34	21-1-34	22-1-34	22-1-34	23-1-34	23-1-34	
	10	Negative pole	10A	16-1-34							Animals alive and well
			10B	16-1-34							
	11	Positive pole	11A	16-1-34	24-1-34	24-1-34	24-1-34	25-1-34	26-1-34	26-1-34	
			11B	16-1-34	24-1-34	24-1-34	24-1-34	25-1-34	26-1-34	26-1-34	
	12	Original brain emulsion	12A	16-1-34	23-1-34	23-1-34	24-1-34	25-1-34	26-1-34	26-1-34	
			12B	16-1-34	23-1-34	23-1-34	24-1-34	25-1-34	26-1-34	26-1-34	

Note —T—Tremor S—Staggering F—Falling L—Lying D—Death

- (5) The material collecting in the positive cell, following electrophoresis of emulsion in sheep's serum of rabies-infected brain, is capable of conveying the disease from rabbit to rabbit, from rabbit to sheep, from sheep to sheep and from sheep to rabbit, with the same certainty as the original emulsions themselves
- (6) This material maintains its virulence for at least 8 days at room temperature

It may be concluded that the virus of rabies—or particles to which it may be attached—carries a negative electrical charge. This being so, the way is open for the separation of this virus from brain tissue and for the preparation of an antirabic vaccine of reasonable bulk.

We desire to express our indebtedness to Colonel Shortt for his cordial co-operation and to Major Iyengar for placing the facilities of the Pasteur Institute, Coonoor, at our disposal.

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Addendum.

This paper had been submitted for publication when the following experiment was carried out. Not having 'gladocol' membranes we attached a filter candle (L3) to the lower end of the side-arm of the positive electrophoresis cell. The filter was immersed in a 5 per cent emulsion in sheep's serum of rabies-infected brain (fixed virus). The current was passed through the apparatus for 24 hours. At the end of this period the buffer filling the cell had become faintly turbid especially in the body of the cell above the agar gel seal. Material was then taken from three locations— from the inside of the filter, from the side-arm of the cell, and, from the body of the cell above the agar seal. These materials were then inoculated subcutaneously into rabbits and sheep, the usual dose of inoculum being employed— 0.2 c.c. for rabbits and 0.5 c.c. for sheep. The results on the tenth day, following inoculation of the animals, were as follows—Material from the interior of the candle caused rabies in 50 per cent of the inoculated animals, that from the side-arm of the cell in 33 per cent of the inoculated animals, that from the body of the cell in 100 per cent of the inoculated animals. Clearly, therefore, the virus of rabies was separated from brain tissue by this means. The greatest concentration of the virus, in the buffer filling the cell, occurred at the point nearest to the positive pole of the apparatus and farthest from the emulsion.

17th February, 1934

NOTICE

Malaria Research and the Malaria Commission of the League of Nations

WE have been asked by the Secretary to the Malaria Commission of the League of Nations to publish the following summary of the work done by the Commission and the programme of investigation recommended for the future

Editor

This year malariologists will have noticed the appearance of several important reports published by the Malaria Commission of the League of Nations, or under its auspices

Organized ten years ago, the Malaria Commission began by studying the epidemiology of malaria as well as all the methods of malaria control employed in different countries in many of which the disease had assumed epidemic proportions as a result of the world war

The first two reports (Malaria Commission, 1925, 1927) therefore deal with the epidemiology of malaria in the countries in which investigations had been carried out on its behalf and on the principles governing the prevention of malaria. The third general report (Malaria Commission, 1933) deals with the therapeutics of malaria and describes the present status of our knowledge of the treatment of the disease and the opinions of the Commission on methods of applying that treatment

A report on 'Housing and Malaria' by Christophers and Missiroli (1933) deals with a problem that was taken up by the Commission in 1928. Another subject, 'Malaria in the Deltas of large Rivers', has been investigated for several years by members of the Commission. Reports have been published on the deltas of the Danube (Zotta, 1932), the Ebro (Pittaluga *et al*, 1932), and the Rhine (Swellegrebel, 1933), followed by a memorandum on certain 'General Considerations' on the problem (Cantacuzene *et al*, 1933)

These different reports define the limits of our present knowledge on these three subjects, but as that knowledge is still far from complete, and malarial research still actively in progress, the Commission feels that lines of further investigation should be indicated in order to point out the problems most urgently calling for solution

The full list of questions on which the Commission recommends that research should be undertaken is given below. It will be noted that all the problems are classified under the three subjects which have been investigated for many years, viz., (i) 'Treatment of Malaria', (ii) 'Housing and Malaria', and (iii) 'Malaria in Deltas'

The study of these problems is not the monopoly of the League Malaria Commission. The members of that Commission feel strongly that they should draw the attention of investigators all over the world to the importance of these problems. No malariologist denies that malaria is essentially a local problem: no country is alike as regards malaria. Therefore, the results of any investigation apply as a rule only to the country where it was made, and if conclusions of a more general value are to be reached, the observations and controlled experiences of different national investigations should first be pooled.

For example, immunity to malaria varies of course, according to the greater or lesser opportunity of repeated infection, to the virulence of the strain, to the number of sporozoites inoculated, and so on. Malaria therapy offers a good opportunity for the study of this problem, but it is of the greatest interest to know if a strain of, let us say, *P. vivax*, which has proved very virulent in England, has the same virulence in Roumania or in Italy, or if a Roumanian strain towards which patients have become immune in Roumania, as the result of repeated inoculation, protects against the English strain and vice versa.

The international co-ordination of research into malaria has proved to be of the greatest value, to carry it out, however, would be difficult, if not impossible, without the existence of an international organization of some sixty members and corresponding experts like the Malaria Commission of the League.

The following is the list of questions recommended for further investigation —

I.—Treatment—clinical and therapeutical research work:—

Determination in hyperendemic and endemic regions of the age groups most seriously affected by the disease and consequently requiring most attention in the matter of treatment. Investigations should cover in the first place the indigenous population and then be extended to immigrants.

Determination of the minimum dose of quinine sufficient for the treatment of the disease in hyperendemic and endemic areas where the natives have attained a certain degree of immunity.

Ascertainment of the dosage (by age groups) of plasmoquine sufficient to prevent the gametocytes from infecting the *Anopheles* and the intervals (per week) at which it should be administered.

Determination of the extent, if any, to which the therapeutical action of quinine is increased by the administration of plasmoquine (combined medication).

A beginning should be made with laboratory research on cases of induced infection before proceeding to apply this method in the field.

Continuation of clinical tests with both types of 'totaquina' in accordance with the method laid down in the report.

Experiments in malaria control by means of medicaments alone without the application of anti-anopheline measures.

Blackwater fever, investigation of the relations between quinine and blackwater fever by means of experiments on animals (malaria in monkeys).

II.—Housing and Malaria:—

Biology and geographical distribution of the different varieties of *Anopheles maculipennis*.

Study of the following species of tropical *Anopheles* from the point of view of a possible differentiation of races —

(a) *A. hyrcanus* and its several varieties (though widely disseminated, this strain is not as a rule very dangerous, though it may become so in certain countries, and especially Sumatra).

(b) *A. biuncatus* (unimportant in Europe, but dangerous in Palestine).

- (c) *A. ludlowi* var *sundaicus* (dangerous everywhere, though there is a great difference between its breeding places on the coast of Java and in the interior of Sumatra)
- (d) *A. gambui* (dangerous everywhere, though its breeding places differ in the Union of South Africa and in Tropical Africa)

Causes of the very great variations met with in the distribution of malaria in certain tropical regions—such as the phenomenon of immune areas in the immediate vicinity of hyperendemic areas—and in particular, the possibility of a relation existing between this phenomenon and the derivation of the *Anopheles*

Investigation of the factors which make certain rice growing areas highly subject to malaria while others remain immune

Study of the African *Anopheles* and the connection between the various species of *Anopheles* and malaria in Africa

III.—Malaria in Deltas —

Initiation or pursuit of research on the varieties of *A. maculipennis* found in European deltas in connection with malarial foci (Danube I bro, Rhine Rhone, Po)

It is suggested that Indian, Siamese and Indo Chinese malarialogists might usefully conduct similar investigations with regard to the races of malaria carrying *Anopheles* in the deltas of their respective countries

Investigation of the influence of agriculture on the domesticity of *Anopheles* and on malarial endemicity

Study of live stock in connection with local anophelism and the disease

Historical study with special reference to malaria of variations in the topographical and demographical characteristics of deltas

Investigation of the degree of susceptibility to malarial infection of inhabitants of deltaic areas

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